Transcription factor binding to, and Regulation of, the HNP-1 *defensin* gene promoter

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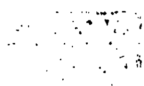
To Mum, Dad, my brothers and sisters, Svet, and the Boss, as well as all the friends who have believed in me throughout my life



"What we observe is not nature itself, but nature exposed to our method of questioning"

Werner Karl Heisenberg (1901-1976)

"I declare that unless otherwise stated, all the work described, both experimental and theoretical was performed by myself."
Pierre Schembri-Wismayer

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Abstract

The defensins are small antibiotic peptides produced during granulocyte differentiation and stored in the azurophilic granules of the mature granulocyte. Defensin is expressed during a brief window in differentiation with mRNA expression peaking at the promyelocyte and early myelocyte stages. Using the promyelocytic leukaemia cell line NB4, nuclear protein binding to the gene sequences immediately upstream of the main transcription start site was studied. Changes in such binding were correlated with mRNA abundance during granulocyte (high defensin) and monocyte (no defensin) NB4 differentiation.

Protein-binding sites, signified by changes in DNAse digestion, were recognised at the ets site at positions -59 and -155, the overlapping c/ebp-myb site at position -120/-105 and at numerous c/ebp-aml sites along the first 240 bp of the upstream sequence.

Binding to most of the sites, with the exception of the -59 ets site, was seen to be considerably greater (by footprinting studies) with granulocytic extract as opposed to undifferentiated NB4 extract. The presence of a DNase 1 hypersensitive site at position –59 (which overlaps the ets site) seems to be essential for defensin expression. The presence of this site appeared to be correlated with baseline expression since it is absent in footprints seen with the extract of monocytic NB4 cells (which do not express defensin). This site (termed footprint α) was shown to bind GABPα, the c/ebp-myb site (within footprint β) was shown to bind C/EBPε whilst the -155 site (in footprint γ) was shown to bind PU.1 by means of electrophoretic mobility shift assays (EMSAs). Different transcription factors were also shown to compete for binding to particular footprinted sites by means of competition EMSAs.

By inserting mutations into the binding sites for these various factors, the ets site at -59 and the c/ebp and myb sites at -120/-105 were shown to be very important for *defensin* promoter activity. For maximal activity in undifferentiated NB4 cells, both these sites were required, but in differentiated cells maximum promoter activity was obtained with a minimal promoter, -67/+15, which did not include any myb sites.

Co-transfection studies showed that C/EBPε and GABPα could up-regulate defensin expression in NB4 cells. GABPβ did not co-operate with GABPα in undifferentiated cells, but synergised with it in differentiated NB4 cells.

In heterologous HeLa cells, defensin promoter activity was stimulated synergistically by C/EBPε with Myb. It was also strongly transactivated by GABPα but such transactivation was unexpectedly inhibited by co-expression of GABPβ. CBFα/β, PU.1 or CHOP-10 were found to co-operate with GABPαβ to stimulate transactivation.

The pattern of transactivation obtained with GABP factors differed from the classical synergism seen between GABPα and GABPβ. These differences may be due to both the different reporter systems being used, and to the defensin promoter in particular, which appears to bind GABPα alone, much more strongly than other promoters such as the *neutrophil elastase* promoter.

The results obtained have been used to create models of possible protein interactions on the *defensin* promoter. Using known patterns of expression of transcription factors during myeloid differentiation, a model is presented describing probable factor interactions responsible for initial up-regulation and later down regulation during differentiation.

Table of Contents

Introduction 1

Chapter 1 : Normal and Malignant Haematopoiesis 1
1.1 Normal Haematopoiesis 1
1.1.1 Proliferation and differentiation in haematopoiesis 2
1.1.2 Lineage commitment in haematopoiesis 2
1.1.3 Neutrophil differentiation 4
1.1.4 Neutrophil Function 5
1.1.5 Granules of the polymorphonuclear neutrophil 6
1.1.6 Defensins 8
1.1.7 Structure and function of the *defensin* genes 9
1.2 Leukaemia 12
1.2.1 Maturation arrest and proliferation 12
1.2.2 Mechanisms of leukaemogenesis 15
1.2.3 Classification of Leukaemia 16
1.2.4 Genetic abnormalities in leukaemia 19
1.2.5 Clinical significance of leukaemia biology 21
1.3 Models for studying haematopoiesis and leukaemia 22
1.3.1 *In vivo* model systems 22
1.3.2 *In vitro* model systems 23
1.3.3 Myeloid cell lines as models of myelopoiesis 24
1.3.4 NB4 as a model of neutrophilic and monocytic differentiation 25

Chapter 2 : Transcriptional regulation of gene expression 26
2.1 A general overview of the elements of transcriptional control 26
2.2 Chromatin and Transcription 27
2.2.1 Nucleosomes 27
2.2.2 Silent and active chromatin 28
2.2.3 Enzymatic modification of chromatin 29
2.3 Sequences involved in the regulation of transcription 30
2.3.1 Promoters and initiators 30
2.3.2 Modularity of promoters 30
2.3.3 Enhancers 31
2.3.4 Locus control regions 31
2.4 Proteins regulating transcription 32
2.4.1 RNA polymerase II 32
2.4.2 General transcription factors 33
2.4.3 TBP and the TBP-associated factors (TAFs) 34
2.4.4 Activators 35
2.4.5 DNA-binding domains 37
2.4.6 Transactivation domains 38
2.4.7 Protein interaction domains 38
2.4.8 Histone Acetyltransferases and other co-activators 40
2.5 Regulation of Transcription factor activity 41
2.5.1 RNA splicing and translation 42
2.5.2 Phosphorylation 43
2.5.3 Proteolysis 43

2.5.4 Ligand binding 44
2.5.5 Multimerisation 45
2.5.6 Nuclear localisation 46
2.5.7 Transcription factor synergism and inhibition 46
2.6 Tissue Specific Gene Transcription 47
2.7 Transcription factors and differentiation 48

Chapter 3 : Transcription factors in haematopoiesis 51
3.1 Transcription factors as effectors in haematopoiesis 51
3.2 Transcription factors involved in myeloid gene regulation 55
3.3 The Myb family of factors 56
3.3.1 c-Myb in haematopoiesis 57
3.3.2 Myb proteins in proliferation and differentiation 57
3.3.4 Myb-target genes 60
3.4 The C/EBP family of transcription factors 60
3.4.1 Functional domains of C/EBP factors 61
3.4.2 C/EBPs as transactivators 63
3.4.3 C/EBPs – expression patterns in different tissues 67
3.4.4 C/EBPs in haematopoiesis 68
3.5 The Ets Family of Transcription factors 69
3.5.1 Ets factors, Development and Cancer 70
3.5.2 The ETS DNA-binding domain 71
3.5.3 Gene Regulation by Ets factors 72
3.5.4 Ets factors in myelopoiesis 75
3.5.5 The PU.1 transcription factor 76
3.5.6 Functional domains of PU.1 77
3.5.7 Transcriptional regulation by PU.1 78
3.5.8 The GA-binding protein 79
3.5.9 Sub-units and domains of GABP 80
3.5.10 Transactivation by GABP 82
3.5.11 Regulation of GABP transcriptional activity 84
3.6 The AML family of Transcription factors 85
3.6.1 AML factors in haematopoiesis 85
3.6.2 AML factors and Cancer 86
3.6.3 Functional domains of the AML factors 87
3.6.4 AML-1 as a transcriptional regulator 88

Aims of the project 90

Materials and Methods 92

Chapter 4 : Materials 92
4.1 Cell lines and tissue culture media 92
4.1.1 Cell lines 92
4.1.2 Media and sterile supplies 92
4.2 Bacterial culture 94
4.2.1 Media and sterile supplies 94
4.2.2 Host cell strains 95
4.3 Plasticware 95

4.4 Water 96
4.5 Chemicals 96
4.6 Nucleotides and polynucleotides 98
4.7 Plasmids 99
4.8 Enzymes and enzyme inhibitors 100
4.9 Antibodies 102
4.10 Membranes, papers and radiographic film 103
4.11 Molecular Biology Kits and Columns 104
4.12 Equipment 105

Chapter 5 : Methods 106
5.1 Cell culture 106
5.1.1 Culture conditions during routine passage of cell lines 106
5.1.2 Cryopreservation and recovery of cells 106
5.1.3 Mycoplasma testing 107
5.1.4 Induction of differentiation 107
5.1.5 Cell Staining - May-Grunwald and Giemsa stains 108
5.1.6 Nitroblue tetrazolium staining 108
5.1.7 Non-specific Esterase staining 109
5.2 Transient Transfections and Reporter Gene Assays 110
5.2.1 Plasmids for Transfections 110
5.2.2 Electroporation 110
5.2.3 Calcium Phosphate transfection 111
5.2.4 Assay for Placental Alkaline Phosphatase 112
5.2.5 RadioImmunoassay for human Growth Hormone 113
5.3 Molecular Biology 114
5.3.1 Microbiological techniques 114
5.3.1.1 Generation of competent cells 114
5.3.1.2 Transformation of bacterial cells 114
5.3.1.3 Preservation of transformed and host strains 115
5.3.2 DNA isolation from bacteria 115
5.3.2.1 Minipreparation of plasmid DNA 115
5.3.2.2 Large scale preparation of DNA - purification by ultracentrifugation 116
5.3.2.3 Large scale preparation of plasmid DNA-purification by QIAgen 117
5.3.3 Recombinant DNA techniques 119
5.3.3.1 Restriction enzyme digestion 119
5.3.3.2 Agarose gel electrophoresis 119
5.3.3.3 Purification of DNA fragments from agarose gels 120
5.3.3.4 Phosphatase treatment of vectors for ligation 121
5.3.3.5 Ligation reactions 122
5.3.4 Quantitation of nucleic acids 122
5.3.5 Sequencing of DNA 123
5.3.6 Polymerase chain reaction (PCR) 123
5.3.6.1 Design of oligonucleotides 125
5.3.6.2 Synthesis of oligonucleotides 126
5.3.6.3 Purification of oligonucleotides 126
5.3.6.4 Site directed mutagenesis by PCR 127
5.3.7 Northern Blot Analysis 129
5.3.7.1 Isolation of cellular RNA 129

5.3.7.2 Electrophoresis of RNA 130
5.3.7.3 Transfer of RNA to membrane 131
5.3.7.4 Random-primed radiolabelling of DNA probes 132
5.3.7.5 Pre-hybridisation and Hybridisation 133
5.3.7.6 Washing and Autoradiography 133
5.3.7.7 Stripping of blots 134
5.3.8 DNAse 1 Footprinting 134
5.3.8.1 Generation of radiolabelled probes 134
5.3.8.2 Isolation of nuclear protein 136
5.3.8.3 G and A tracking reactions 137
5.3.8.4 Footprinting reaction 138
5.3.8.5 Gel analysis 139
5.3.9 Electrophoretic Mobility Shift Assay(EMSA) 140
5.3.9.1 Preparing double stranded oligonucleotides 140
5.3.9.2 Radiolabelling of probes 141
5.3.9.3 Isolation of nuclear protein 142
5.3.9.4 Incubation reaction and gel analysis 142
5.3.9.5 Modifications of EMSAs including supershift analysis 143
5.3.10 Western Blot Analysis 144
5.3.10.1 Isolation of nuclear protein 144
5.3.10.2 Quantification of samples 145
5.3.10.3 SDS-Polyacrylamide gel analsis (PAGE) 145
5.3.10.4 Wet Blotting 146
5.3.10.5 Protein detection 147
5.3.11 In Vitro Transcription/Translation 148

Results 149

Chapter 6: Characterisation of the NB4 cells 149
6.1 Differentiation of NB4 cells - Morphological Changes 149
6.2 Functional Evidence of Granulocytic differentiation 152
6.3 Functional Evidence of Monocytic Differentiation 155
6.4 *Defensin* expression during NB4 cell differentiation 157

Chapter 7 : *In vitro* protein binding to the *defensin* promoter 162
7.1 DNAse 1 footprinting 162
7.1.1 Changes in footprints on the defensin promoter with differentiation... 162
7.1.2 Preliminary interpretation of Footprinting Results 171
7.2 Electrophoretic Mobility Shift Sssay on the defensin promoter 174
7.2.1 Different protein binding with differentiation 174
7.2.2 Individual footprint sequences cannot compete promoter-bound complex 176

Chapter 8 : Analysis of Footprint-bound proteins by EMSA 179
8.1 Analysis of FPα-binding proteins 179
8.1.1 Changes in FPα binding with differentiation 179
8.1.2 Characterisation of FPα binding by competition EMSAs 181
8.1.4 GABP binds to FPα 184
8.2 Analysis of FPβ-bound proteins 187
8.2.1 Changes in binding to FPβ with differentiation 187

8.2.2 Characterisation of FPβ binding by competition EMSAs 189
8.2.3 C/EBPε and GABPα bind to FPβ in granulocytic cells 191
8.3 Analysis of FPγ-bound proteins 194
8.3.1 Changes in FPγ binding with differentiation 194
8.3.2 Characterisation of FPγ binding by competition EMSAs 194
8.3.3 PU.1 binds to FPγ 198
8.3.4 C/EBPε binds to FPγ as well as to FPα 201

Chapter 9 : Factor interactions on the *defensin* promoter 204
9.1 Antibodies alter NB4 nuclear protein binding to the *defensin* promoter 204
9.2 Anti-Ets factor antibodies increase HL60 nuclear factor binding to FPγ 207
9.3 Increased NB4 nuclear protein binding to FPα upon competition 209
9.3.1 Low mobility complex binding after prolonged competition 209
9.3.2 Increased FPα binding after competion with Ets-binding oligonucleotides is due to C/EBPε 211
9.3.3 Ets-related complexes bind more strongly to FPα after C/EBP removal 215

Chapter 10 : Western analysis - Changes in transcription factor abundance with differentiation 216

Chapter 11 : Characterisation of the reporter gene system for transfection studies 219
11.1 Anomalous up-regulation of empty growth hormone reporter vector 220
11.2 Modification of the growth hormone reporter vector 220
11.3 Differential responses with new and old hGH vectors 224
11.3.1 Different vector responses to differentiation and co-expressed activators 224
11.3.2 Different activity of promoter fragments in old and new hGH vectors 224
11.4 Optimising the new hGH reporter test system 228
11.4.1 Optimisation of transfection conditions 228
11.4.2 Control studies with uninduced, ATRA-exposed and differentiated cells 229

Chapter 12 : Transfection studies in NB4 cells 233
12.1 Up-regulation of *defensin* promoter activity upon differentiation 233
12.2 Mutation of C/EBP-, Myb- and Ets-binding sites reduce *promoter* activity 237
12.3 Co-transfection studies with various activators in NB4 cells 240
12.4 GABP up-regulates the *defensin* promoter diversely, on differentiation 242
12.5 Effects of site-specific and deletion mutations on transactivation by co-activators 243

Chapter 13 : Promoter studies in non-myeloid HeLa cells 246
13.1 Myb and C/EBPε synergistically transactivate the *defensin* promoter 246
13.2 C/EBP α and β do not co-operate with c-Myb and can be inhibited by CHOP-10, unlike CEBPε 248
13.3 GABPα strongly activates the *defensin* promoter without GABPβ 251
13.4 PU.1 co-operates with GABPαβ but not with GABPα in transactivating the *defensin* promoter 254
13.5 Additive and co-operative transactivation of the *defensin* promoter by different transcription factors 259

Chapter 14 : *In vitro* binding studies with HeLa nuclear proteins........ 263
14.1 GABP proteins in HeLa cells -Westerns and EMSAs........ 263
14.2 GABPα forms a complex other than GABPαβ with HeLa extract........ 265
14.3 A C/EBP-containing complex binds to FPα in the presence of GABPβ.... 267
14.4 GABP sub-units bind to the minimal *defensin* promoter as........ complexes different from GABPαβ........ 269

Discussion........ 272

Chapter 15 : Critiques of *in vitro* and transient transfection experiments........ 272
15.1 NB4 and HeLa cells as model systems for studying *defensin* gene regulation........ 272
15.2 *In vitro* footprinting assay........ 273
15.3 Electrophoretic mobility shift assays (EMSAs)........ 274
15.3 Transient transfections for studying promoter activity........ 276
15.4 Reporter gene systems and promoter analysis........ 277

Chapter 16 : Analysis of defensin promoter binding and activity........ 279
16.1 Factors binding to FPα........ 279
16.2 Factors binding to FP β........ 283
16.3 Proteins binding to FPγ........ 285
16.4 Competition and/or co-operation between different sites in the same promoter........ 288
16.5 C/EBPε and GABP regulate *defensin* expression in NB4 cells........ 289
16.6 GABP-dependent *defensin* transactivation is enhanced by NB4 cell myeloid differentiation........ 290
16.7 Myb and C/EBPε synergistically trans-activate the *defensin* promoter...... 293
16.8 GABP proteins may interact physically and functionally with alternative partners........ 295
16.9 Incongruities between exogenous and endogenous promoter activation.... 297

Chapter 17 : Models of *defensin* promoter interactions........ 299
17.1 Model of transcription factor interactions on promoter deletion mutants transfected into NB4 cells........ 299
17.2 Interactions on the *defensin* promoter in HeLa cells........ 303
17.3 Stage-specific expression of *defensin* as a function of changes in the abundance of various transcription factors........ 306

Chapter 18 : General conclusions and future work prospects........ 310
18.1 Completing the picture - EMSAs and further functional analysis of the minimal promoter........ 310
18.3 Understanding intra-promoter binding site competition and co-operativity 311
18.3 Further analysis on the luciferase and growth hormone reporter genes....... 312
18.4 Could GABP sub-unit imbalances result in the abnormal gene transcription?........ 313

References........ 315

List of figures

Figure Number	Figure Title	Page
Figure 1	Haematopoiesis.	3
Figure 2	Chronological expression of different myeloid granule proteins.	7
Figure 3	Mechanism of leukaemogenesis.	13
Figure 4	Anatomy of a transcription factor complex.	36
Figure 5	Growth factors and transcription factors important in haematopoiesis.	52
Figure 6	ATRA-induced maturation of NB4 cells.	150
Figure 7	Vitamin D3 and Phorbol Myristate Acetate (PMA) – induced monocytic differentiation of NB4 cells.	151
Figure 8	Changes in reducing ability during granulocytic NB4 cell differentiation.	153
Figure 9	Assessing granulocytic differentiation by quantifying the amount of cells reducing NBTZ.	154
Figure 10	Non-specific esterase activity in NB4 cells induced to differentiate to monocytes.	156
Figure 11	Measuring monocytic differentiation by quantification of the percentage of esterase.	158
Figure 12	Northern blots of total RNA during chemically-induced granulocytic and monocytic NB4 cell differentiation.	159
Figure 13	Increased mRNA accumulation with granulocytic differentiation	160
Figure 14	DNAse1 footprinting of the defensin upstream sequence – 240/+15 (antisense strand).	163
Figure 15	DNAse1 footprinting of the defensin upstream sequence – 240/+15 (sense strand).	164
Figure 16	DNAse1 footprinting of the defensin –240/+15 upstream sequence using 40μg of nuclear extract with 0.5U of DNAse1.	166

Figure 17	Diagrammatic representation of defensin promoter DNAse1 footprinting experiments with 40μg of nuclear protein extract.	167
Figure 18	Comparison of the DNAse1 protection (footprinting) seen with different amounts of nuclear proteins.	169
Figure 19	Diagrammatic representation of the differences in footprinting studies with different amounts of nuclear protein extract.	170
Figure 20	Electrophoretic mobility shift assay (EMSA) of the defensin upstream sequence -240/+15.	175
Figure 21	Competition EMSA with -240/+15 promoter fragment as the [γ-^{32}P]-labelled probe sequence.	178
Figure 22	Changes in nuclear protein binding to FPα with differentiation.	180
Figure 23	Competition EMSA of FPα-bound proteins.	182
Figure 24	Competition EMSA to assess AML and GABP binding to FPα.	183
Figure 25	GABP binding to FPα.	185
Figure 26	Changes in binding to FPβ with differentiation.	188
Figure 27	Identification of FPβ binding proteins by competition EMSA	190
Figure 28	Identification of FPβ-binding proteins by means of antibody supershifts.	193
Figure 29	Changes in binding to FPγ and to ets-site-mutated FPγ, with differentiation.	195
Figure 30	Identification of proteins binding to FPγ by competition EMSAs.	197
Figure 31	Ets protein binding to FPγ.	199
Figure 32	C/EBPε binding to FPα and FPγ.	202
Figure 33	Identification of proteins binding to the -240/+15 defensin upstream sequence.	205
Figure 34	Increased binding of HL60 proteins to FPγ upon co-incubation with anti-Ets factor antibodies.	208

Figure 35	More FPα competition EMSAs.	210
Figure 36	Enhanced binding of undifferentiated NB4 nuclear proteins to FPα following oligonucleotide competition.	212
Figure 37	Enhanced binding of granulocytic NB4 nuclear proteins to FPα following oligonucleotide competition.	214
Figure 38	Western analysis of changes in transcription factor abundance with differentiation.	217
Figure 39	Transactivation of the empty genomic growth hormone reporter vector.	221
Figure 40	Corrective measures applied to the genomic growth hormone reporter vector.	223
Figure 41	Up regulation of old and new growth hormone reporter vectors by differentiation and transactivators.	225
Figure 42	Different relative promoter activity of defensin promoter deletion mutant-driven reporter constructs.	227
Figure 43	Identifying the best time to withdraw medium for testing reporter activity.	230
Figure 44	Assessing growth hormone production on differentiation.	231
Figure 45	Functional activity of *defensin* promoter constructs in undifferentiated and differentiated NB4 cells.	234
Figure 46	Effects of mutating transcription factor binding sites on promoter activity.	238
Figure 47	Changes in transactivation and transcription factor synergism with differentiation.	241
Figure 48	Transactivation of mutated *defensin* gene promoters in uninduced NB4 cells by GABPαβ and C/EBPε.	244
Figure 49	Transactivation of *defensin* gene promoters in HeLa cells by MYB and C/EBPε.	247
Figure 50	Effects of CHOP on Myb-C/EBP-induced transactivation of the *defensin* promoter.	250
Figure 51	Transactivation of *defensin* mutant promoter constructs in HeLa cells by GABPs.	252
Figure 52	Transactivation of the *cd18* minimal promoter.	255

Figure 53 Co-operative transactivation of *defensin* mutant promoter constructs in HeLa cells by PU.1 and GABPαβ. 256

Figure 54 Increased amounts of PU.1-expressing vector repress GABPαβ-induced transactivation. 258

Figure 55 Co-operative transactivation of the defensin promoter in HeLa cells by different sets of transcription factors. 261

Figure 56 GABP proteins in HeLa cells and their binding to the defensin promoter. 264

Figure 57 Formation of complexes other than GABPαβ between GAI sub-units and HeLa nuclear proteins. 266

Figure 58 GABPα and β form complexes of the –70/+15 defensin promoter fragment with HeLa proteins, distinct from GABPαβ. 270

Figure 59 Model for FPα competition EMSAs. 282

Figure 60 Model of DNA-protein interactions on FPβ and FPγ. 286

Figure 61 Model of GABP-dependent transactivation in NB4 cells. 292

Figure 62 Model of transcription factor binding to defensin promoter deletion mutants. 300

Figure 63 Model showing interactions between the GABPα and β sub-units and other factors. 305

Figure 64 Model to show the up regulation and subsequent down regulation of the defensin promoter with differentiation. 307

List of Tables

Table Number	Title of Table	Page
Table 1	The French American British (FAB) classification of Acute Myeloid Leukaemias	18
Table 2	Commoner known translocations and associated genetic rearrangements found in acute childhood leukaemias	20
Table 3	Transcription factor DNA-binding domains	39
Table 4	Effects of transcription factor disruption by homologous recombination on haematopoetic development in the mouse	53
Table 5	Transcription factors interacting with C/EBP factors and some of the target genes co-operatively transactivated by such interacting combinations	65
Table 6	Physical and Functional co-operative interactions between different Ets factors and other transcription factors of diverse families	74

Abbreviations

1,25 Vit D3	1,25 dihydroxy-cholecalciferol
A	Adenosine
aa	Amino acid
Ab/s	Antibody/ies
Ac	Acetate
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloblastic leukaemia
AML-1	Acute myeloid leukaemia 1 (transcription factor)
APS	Ammonium persulphate
ATRA	All-*trans* retinoic acid
BFU	Burst-forming unit
bHLH	Basic helix-loop-helix
bp	Base pair
BSA	Bovine serum albumin
bzip	Basic zipper protein
C	Cytosine
c-	cellular
C/EBP	CCAAT/enhancer-binding protein
CBF	Core binding factor
CBP	CREB-binding protein
cDNA	complementary deoxyribonucleic acid
CFU	Colony-forming unit
$CHCl_3$	Chloroform
Ci	Curie
CLL	Chronic lymphocytic leukaemia
CML	Chronic myelomonocytic leukaemia
CSF	Colony-stimulation factor
ddH2O	Double distilled water
del(x)	deletion
DEPC	Diethylpyrocarbonate
dH2O	Distilled water
DMSO	Dimethylsulphoxide

DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	3' deoxyribonucleoside 5' triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid, disodium salt
EMSA	Electrophoretic mobility shift assay
Epo	Erythropoietin
ETS	*Ets*-binding domain
FCS/FBS	Foetal calf serum/Foetal bovine serum
FP	Footprint
G	Guanine
GABP	Guanine-Adenine-binding protein
GAPDH	Glyceraldehyde phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
hGH	Human growth hormone
HLH	Helix-loop-helix
HSV	Herpes simplex virus
IL-3	Interleukin-3
inv(x)	inversion
kD	Kilo dalton
LB	L-broth
LTR	Long terminal repeat
MAP kinase	Mitogen activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MIP-1α	Macrophage inhibitory protein-1α
MOPS	3-(N-morpholino) propanesulphonic acid
mRNA	Messenger ribonucleic acid
mSCF	Transmembrane form of stem cell factor
NE	Neutrophil elastase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered-saline
PCI	Phenol:chloroform:isoamylalcohol
PCR	Polymerase chain reaction
PEBP	Polyoma enhancer binding protein

PI	Pre-immune
PMSF	Phenylmethylsulfonyl fluoride
poly(dI-dC)	Polydeoxyinosinic deoxycytidylic acid
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulphate
SFFV	Spleen focus-forming virus
SLM	Special liquid medium
Sp1	Promoter-specific transcription factor-1
SRF	Serum-response factor
T	Thymine
t(x;x)	translocation
TBP	TATAA-binding protein
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline- Tween 20
TEMED	Tetramethylenediamine
tk	Thymidine kinase
TNT	Coupled *in vitro* transcription and translation reactions
TPA/PMA	Tetra decanoyl phorbol acetate/ Phorbo Myristate acetate
Tris	2-amino-2-(hydroxymethyl) propane-1,3-diol
tRNA	Transfer ribonucleic acid
Tween 20	Polyoxyethylene sorbitan monolaurate
U	Units
UV	Ultra violet
V	Volts
v-	Viral
v/v	Volume for volume
Vit.D3	cholecalciferol
w/v	Weight for volume

Introduction

Chapter 1 : Normal and Malignant Haematopoiesis

1.1 Normal Haematopoiesis

Haematopoiesis is the process whereby blood cells, which perform different functions in mammalian blood, are continuously produced from precursor cells and ultimately from multi-potential stem cells (1).

In mammals, haematopoiesis occurs first, as yolk sac /embryonic (or primitive) haematopoiesis and later as foetal/adult (or definitive) (2;3). These two processes are similar in certain ways as in the production of red blood cells and also macrophages. Primitive haematopoiesis differs from definitive haematopoiesis in the lack of formation of the other lineages. Whilst embryonic haematopoiesis occurs in the yolk sac, adult haematopoiesis is now known to commence in a region of mesoderm known as the aorta-gonodal-mesonephric (AGM) region (4). It appears that this definitive haematopoiesis is not due to stem cells from the yolk sac colonising the AGM region but is a separate process with *de novo* formation of stem cells.

Such stem cells are described arbitrarily by their capability to reconstitute long term lympho- and myelo-poiesis *in vitro* or in an experimental animal, which has been irradiated so as to ablate it's own blood forming tissue (5). Whilst their exact phenotype has not been conclusively identified, they make up part of a population of cells which are CD 34+ CD33- CD38- HLA DR- Thy-1^{lo} and are negative for all lineage markers (6). Differentiation of these stem cells down different pathways results in the formation of eight cell lineages, which make up mammalian blood (Figure 1).

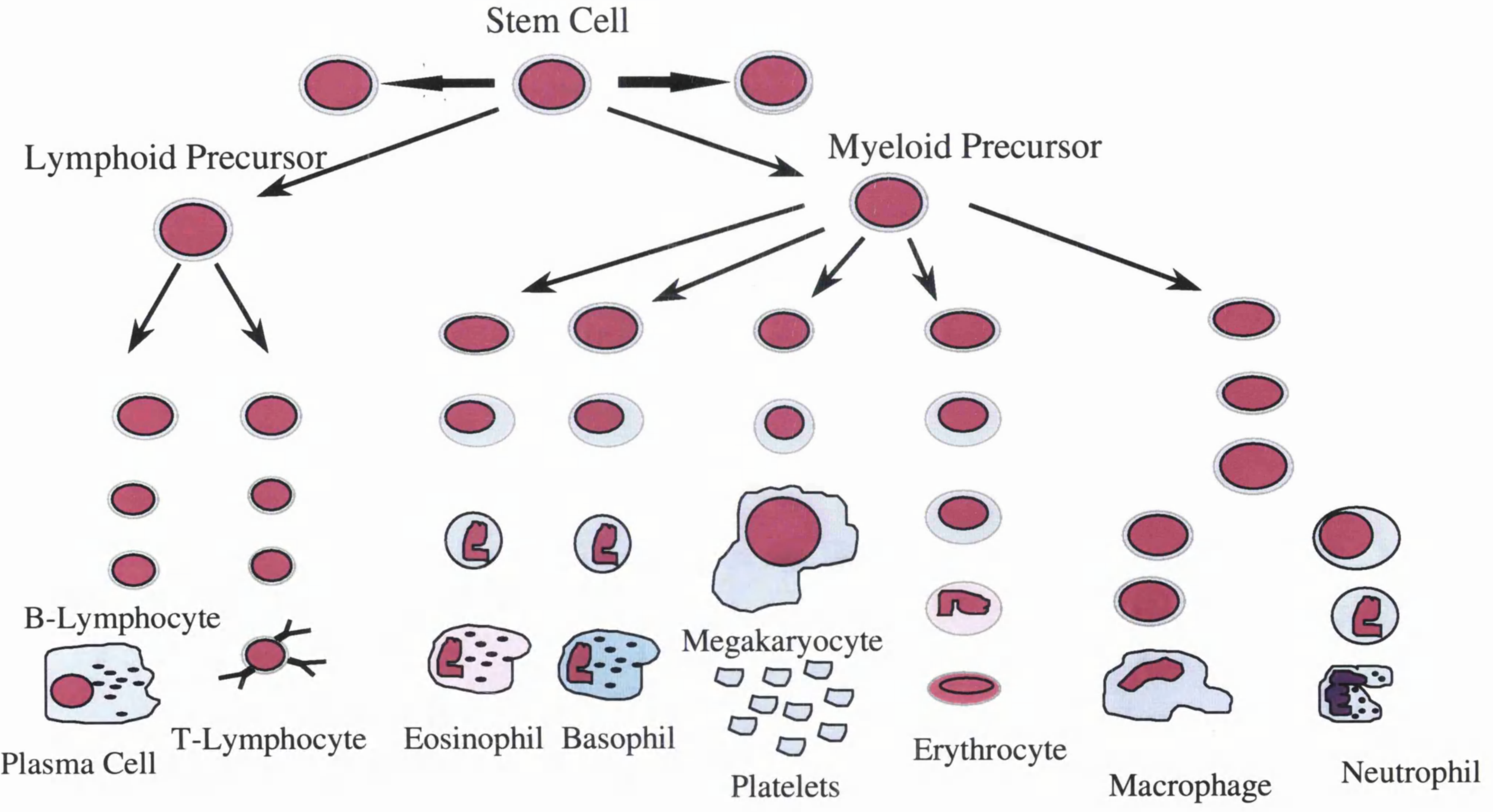
1.1.1 Proliferation and differentiation in haematopoiesis

In many other mesodermal tissues (muscular or cartilaginous or adipose tissue), commitment of progenitor cells to a specific lineage is often associated with a loss of cell division (7). On the other hand, haematopoietic development into different lineages requires taking decisions to differentiate whilst still in a state of high proliferative potential (8). Despite this, there is still a clear inverse correlation between the proliferative potential of haematopoietic cells and the extent of their differentiation (9). Whilst cessation of proliferation is not a virtual pre-requisite for commitment to haematopoietic differentiation as it is in many other tissues, changes in cell cycle do occur, concomitant with differentiation-related decisions. An increase in the G1 phase of the cell cycle during erythropoietin-induced erythroid differentiation is a case in point (10).

1.1.2 Lineage commitment in haematopoiesis

Whether the process of haematopoietic lineage commitment is primarily a stochastic or a directed process has long been a cause for debate (11). Some rather elegant work by Fairbairn et al (12), has shown that by inhibiting cell death, single cells taken from a multi-potential cell line can mature into cells of different lineages without the addition of any conditioning growth factors or cytokines. This argues in favour of a stochastic process. On the other hand it is quite clear that the differentiation of both cell lines and primary stem cells can be directed towards one or another lineage by the presence of different growth factors (13). Recent interesting studies using the reverse-transcriptase polymerase chain reaction (RT-PCR) has shown that a single cell will

Fig. 1 Hematopoiesis.
Cells from the self-replicating stem cell compartment, can differentiate into progenitors of lymphoid or myeloid lineages. Through further maturation, end-stage cells of the eight major mature blood lineages are formed (other lineages such as the Natural Killer cells, are not shown).


Stem Cell
Lymphoid Precursor
Myeloid Precursor
B-Lymphocyte
Plasma Cell
T-Lymphocyte
Eosinophil
Basophil
Megakaryocyte
Platelets
Erythrocyte
Macrophage
Neutrophil

express genes found in different lineages rather promiscuously prior to a definitive commitment and subsequent maturation towards one particular lineage (14). These different studies together suggest that haematopoietic stem cells are primed to differentiate down a number of possible pathways and that either stochastically or under the influence of growth factors, (and probably environmental cell-cell interactions) they mature into fully differentiated cells of one or other lineage. Any commitment decisions are associated with expression of one or more lineage-restricted transcription factors which also tend to auto-regulate their own expression in order to reinforce the decision (15;16). Once such a commitment has taken place, it is relatively irreversible and co-expression of transcription factors required for the development of a different lineage may inhibit maturation or even cause apoptosis (17). The role of transcription factors in haematopoiesis is discussed in greater detail later on.

1.1.3 Neutrophil differentiation

The neutrophil granulocyte, the second most common blood cell in a healthy human adult, is the end stage cell of one of the myeloid lineages. Early progenitor cells differentiate along one of two major lineages, namely the lymphoid and the myeloid lineages (18). Colony forming assays using bone marrow or foetal liver stem cells identify single colonies containing granulocytes, erythrocytes, monocytes and megakaryocytes (19). Such colonies are thought to originate from a pluri-potential myeloid precursor named the CFU-GEMM (Colony Forming Unit-Granulocytes, Erythrocytes, Monocytes and Megakaryocytes). Such cells are early neutrophil precursors. With continuing maturation along the neutrophil lineage, precursors become

restricted to forming only granulocytes and monocytes (Such a cell has been named a CFU-GM). This can further differentiate from myeloblast to promyelocyte at which point the precursor is still bi-potential and capable of forming both neutrophil granulocytes and monocytes. As maturation progresses along the neutrophil lineage into myelocytes, and metamyelocytes, the cell is now committed to form a neutrophil and looses its capability of cell division as it forms a band cell and eventually a mature polymorphonuclear granulocyte (20). Throughout the process of differentiation from the earlier cells, there is a progressive reduction in the nuclear cytoplasmic ratio associated with chromatin condensation. In the later stages, as the cell becomes committed to the granulocyte lineage, different types of granules appear in the cytoplasm of the cell (21).

1.1.4 Neutrophil Function

The neutrophils, found at a concentration of $4 - 11 \times 10^9$/L in human blood, are the primary defence of the body against infecting micro-organisms. Whilst not as specifically targeted as the lymphocytes they do not require a prior exposure to an invading pathogen and therefore whilst being a relatively non-specific component of the immune system defences, their response is more rapid on occasion. As well as the immediate response whereby these cells home in to sites of inflammation or injury (including by extravasation into the tissues), and phagocytose injured cells and pathogens, neutrophils also secrete chemotactic factors. These attract antigen-presenting cells to the sites of infection and in this manner help bolster the more specific lymphocyte-dependent immune response (22).

Neutrophils destroy infecting organisms by one of two mechanisms once these have been phagocytosed. These are oxygen-dependent and oxygen-independent mechanisms (23). Oxygen-dependent mechanisms involve the use of reactive oxygen species (including hydroxyl radicals, hydrogen peroxide and superoxide anions) which are produced as a result of the activity of an NADPH oxidase assembled on neutrophil cytoplasmic membranes (24). Non-oxidative mechanisms depend on peptide antibiotics with a broad anti-microbial activity found within the different neutrophil granules (25).

1.1.5 Granules of the polymorphonuclear neutrophil

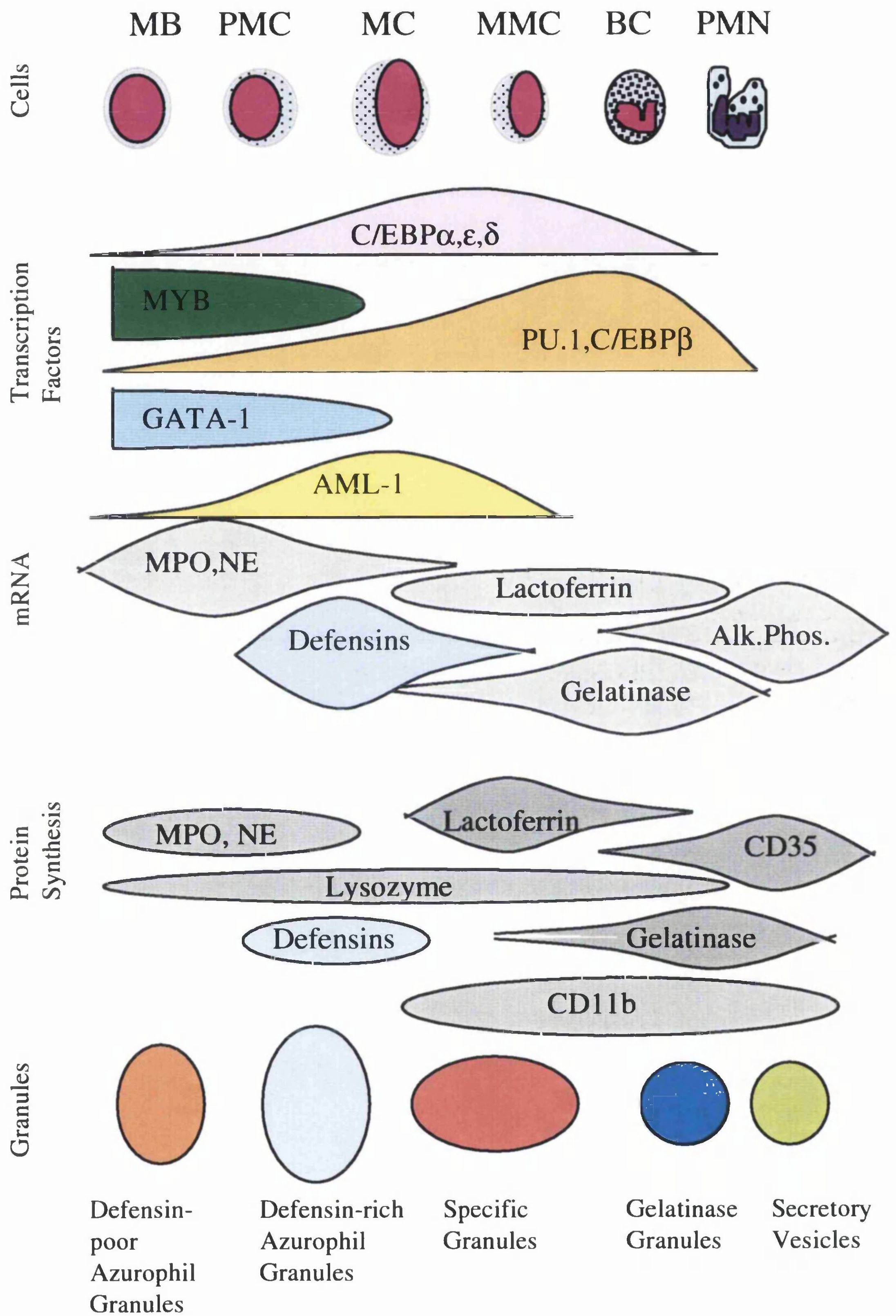
The mature neutrophil contains primary or azurophilic granules (so named due to more intense staining with the azure component of cellular stains), secondary or specific granules, gelatinase granules and secretory vesicles. These different granules appear at different points during the maturation of the

(26;27). Primary granules are already apparent as early as the late myeloblast and promyelocyte stages whilst specific granules, gelatinase granules and secretary vesicles develop in the more mature myelocytes, metamyelocytes and band cells respectively. Some of the different proteins present in these granules, together with a time frame showing their time of appearance, are seen in Figure 2 - modified from (21).

Neutrophil granule proteins are sorted to the cytoplasmic granules by processes common to all cells (28). Rather elegant studies whereby neutrophil proteins are expressed, driven by different tissue-specific or constitutive promoters show that the

localisation of a particular protein to one or other granule is related to the time point in differentiation when the gene is predominantly expressed. Thus neutrophil gelatinase

Fig.2 Chronological expression of different myeloid granule proteins. The figure shows the correlation between granule protein synthesis and the transcription factors present at different stages of myeloid precursor differentiation towards neutrophils. MB-myeloblast, PMC-promyelocyte, MC-myelocyte, BC-band cell, PMN-polymorphonuclear cell (neutrophil).


MB
PMC
MC
MMC
BC
PMN
Cells
Transcription Factors
C/EBPα,ε,δ
MYB
PU.1,C/EBPβ
GATA-1
AML-1
mRNA
MPO,NE
Lactoferrin
Defensins
Alk.Phos.
Gelatinase
Protein Synthesis
MPO, NE
Lactoferrin
CD35
Lysozyme
Defensins
Gelatinase
CD11b
Granules
Defensin-poor Azurophil Granules
Defensin-rich Azurophil Granules
Specific Granules
Gelatinase Granules
Secretory Vesicles

associated lipocalin (NGAL) was found to be localised to the azurophilic granules together with myeloperoxidase (whilst it is normally found in the specific granules with lactoferrin) when it is transcribed under the control of a constitutive viral promoter in myeloblast HL60 cells (29).

Neutrophil granules have different functions with primary granule proteins only being liberated into the phagocytic secondary lysosome whilst secondary granule proteins are also expressed in the cytoplasm and secretary granule proteins are secreted into the extra-cellular space (30;31). For this reason, in order for normal function of the neutrophil, it is essential that such proteins are accurately localised and therefore their appropriate stage specific expression during differentiation is of paramount importance.

1.1.6 Defensins

The defensins are small cysteine-rich cationic peptide molecules (29-35 amino acids in length) with a broad anti-microbial activity (against Gram positive and Gram negative bacteria, fungi and certain enveloped viruses) which are found in the neutrophil azurophilic granules where they represent up to 50% of the total proteins (32). Defensins are synthesised as inactive pre-pro-peptides about 94 amino acids in length. Cleavage of the signal sequence results in an inactive pro-peptide of 75 amino acids localised primarily to the cytosol. Once localised to the granules, the anionic pro-peptide sequence is cleaved off by a proteolytic process resulting in the active antibiotic molecule (33). Unlike the contents of the secondary granules, defensins are not secreted to any extent by the neutrophil but are released into phagosomes by fusion, thus forming a secondary lysosome (34). Even if any are released into the environment, they are

rapidly neutralised by various serum components (35). This ensures that the defensin antibiotics are active and present in sufficient concentrations only within phagocytic vacuoles. This is essential because defensins which exert their antibiotic effect by permeabilising target cell membranes (36), are also cytotoxic to host cells (37). Apart from their direct cytotoxic effect, some defensins can act as opsonins or as chemo-attractants to monocytes (38).

Within the haematopoietic system, no other cell type expresses *defensin* genes. They are however are also expressed by intestinal epithelial Paneth cells, which secrete them into the intestinal lumen thereby producing another defence against infection within the intestinal crypt (39). Another class of defensins with a somewhat different peptide structure known as beta defensins have also been found in the epithelial cells of the salivary glands (40), ocular conjunctiva (41), respiratory passages (42) and placenta (43). These are all different sites where a defensive barrier against invading organisms is essential.

1.1.7 Structure and function of the *defensin* genes

A number of *defensin* genes and *defensin*-like genes are found in the human genome and expressed in different tissues. The genes expressing the beta defensin, HBD-1 and the alpha defensin human neutrophilic peptide 1(HNP-1), are located in close proximity with 150kb of each other on the short arm of chromosome 8 suggesting a common evolutionary derivation despite considerable sequence differences (44). Amongst the alpha or classical *defensin* genes, there are differences in the structure between genes expressing the neutrophil peptides HNP1-4 and the intestinal human defensins (HD)-5 and 6 (45). The neutrophil-expressed genes have three exons and two

introns, the first exon coding for an untranslated 5' sequence, the second exon the translation start site, signal sequence and the pre-pro-peptide sequence removed during post-translational processing, and the last exon codes for the effective peptide. The intestinal expressed *defensin* genes have two exons only lacking the untranslated sequence. Regulatory regions are functionally different between the two genes as would be expected by their patterns of expression. The sequence homologous to the first intron of the HNP-1 *defensin* gene acts as the promoter for the HD-5 *defensin* gene (45). HNPs1-3 are expressed in a co-ordinated manner in neutrophils. HNP-4 protein on the other hand is present at about a 50-fold lower concentration than HNPs 1-3(46). HNP-1 and -3 cDNAs differ at just 2 nucleotides despite being coded for by separate genes, whilst HNP-2 protein which lacks the N-terminal amino acid is probably also coded for by one of the previously mentioned genes (47;48). The HNP-4 *defensin* gene differs somewhat from the HNP-1 defensin gene, having about 72% identity to the latter as well as an extra 83 base segment (49).

Earlier studies both in our laboratory and in others (50;51), found that *defensin* mRNA is expressed in normal bone marrow and in patients with myelocytic leukaemia but not in normal mature granulocytes. In situ hybridisation studies of bone marrow populations showed that *defensin* mRNA is present mostly in promyelocytes and myelocytes with lesser amounts detected in myeloblasts and metamyelocytes indicating that transcription is restricted to a short window during differentiation.

In recent studies monocytic differentiation of the HL60 myeloblastic cell line, with 12-O-tetradecanoyl phorbol 13-acetate (TPA), was shown to result in the rapid loss of defensin mRNA (52). On the other hand, granulocyte differentiation using the chemical inducer all-*trans* retinoic acid (ATRA), markedly up-regulates *defensin* mRNA (around 80-fold by 1μm ATRA). Maximum up-regulation occurs four days after

initiation of induction with *defensin* mRNA declining to the uninduced levels thereafter. Doses incapable of inducing morphological differentiation (as assessed by nitroblue tetrazolium reduction) were still capable of up-regulating defensin expression. Despite this, ATRA-induced *defensin* gene up-regulation is probably not due to direct activation by ligand-bound RARα protein since it showed a more delayed pattern of kinetics and required de novo protein synthesis as confirmed using cyclohexamide. The lack of any strict consensus RAREs (Retinoic acid receptor elements) within the immediate upstream sequence studied also argues against such a direct effect. ATRA did not appear to be causing any stabilisation of *defensin* mRNA whilst nuclear run-on assays indicated that much (or possibly all) of the observed increase in *defensin* mRNA was due to an increase in transcription which reaches fourfold by day1 post-induction. The later down regulation is probably also due to a reduction in the rate of transcription. Interestingly, G-CSF synergises with ATRA to produce a more rapid accumulation of defensin message peaking at 48 hours.

Whilst retinoic acid receptors may not be directly responsible for defensin up-regulation, a number of transcription factors have very recently been shown to bind to and/or up-regulate the defensin promoter in mammalian cells. These include C/EBPα and AML-1which up-regulate the mouse defensin promoter (53), and a putative Ets factor which binds a defensin promoter ets site in a phosphorylation dependent manner (54).

1.2 Leukaemia

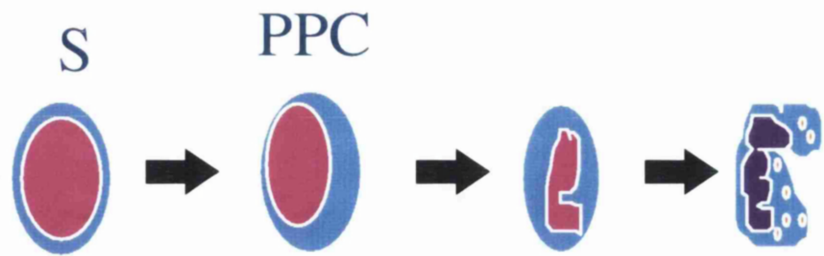
Leukaemia, literally meaning white blood, is a malignant disorder identified by an increased amount of leukocytes or white blood cells in the blood. The leukaemias are a heterogeneous group of diseases, which have been classified characteristically according to cellular (morphological), and clinical features and more recently through immunophenotyping (55). Certain molecular abnormalities however (particularly the Philadelphia chromosome in chronic myeloid leukaemia), have long been recognised as pathognomonic for certain types of disease (56).

1.2.1 Maturation arrest and proliferation

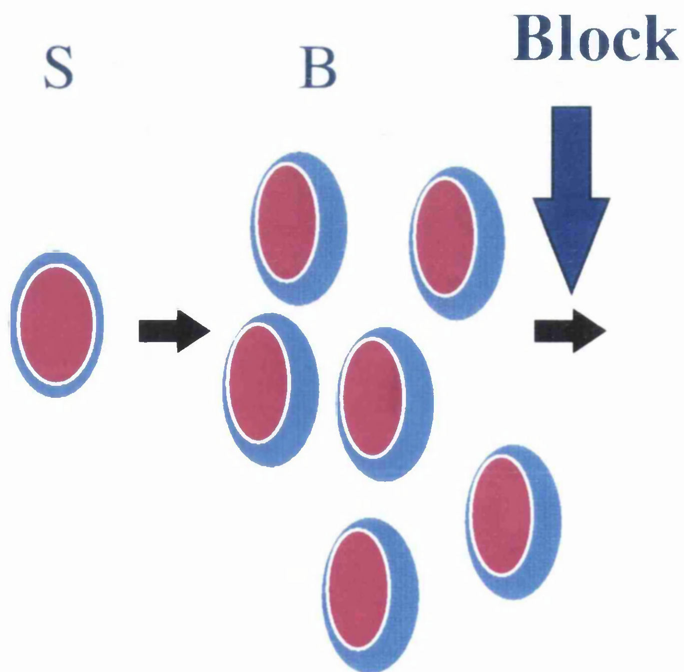
Leukaemia can be defined as the uncontrolled proliferation or expansion of a clone of leukocyte precursors that do not retain the capacity to differentiate normally to mature blood cells (see Fig.3). The morbidity in patients with leukaemia is often not directly due to the clonal proliferation in itself but due to the fact that the uncontrolled growth of this clone of cells competes with and suppresses the normal haematopoietic process. This results in a lack of adequate production of the normal functional mature blood cells causing defective clotting, anaemia and problems with infection.

Pre-leukaemic conditions, which are not actually leukaemia, may manifest either as uncontrolled proliferation (myeloproliferative syndromes) or as abnormal differentiation (myelodysplasia) but both proliferation and maturation arrest are required for leukaemia to develop (57). In either of these pre-leukaemic conditions, a second complementing defect can transform the disease into leukaemia.

Fig.3 Mechanism of leukaemogenesis. Top panel – Normal haematopoiesis where stem cells (S) differentiate into pluri-potential progenitor cells (PPC) and from then through different stages into mature neutrophils. Bottom panel – myelodysplasia with the diseased stem cell clone (S) also differentiates into a proliferative progenitor cell which is however blocked in its capability to mature normally. These cells, called blasts (B), which are still capable of proliferation, continue to replicate (possibly developing other defects and mutations which further enhance their proliferative potential). This expanding clone of cells eventually begins to compete with the normal stem cells causing the characteristic bone marrow failure of leukaemia.



Haematopoiesis



Leukaemopoiesis

The requirement for both the proliferative and the maturation arrest components in the process of leukaemogenesis can be seen both in natural disease states as well as in experimental systems. One mechanism whereby a stem cell or proliferative progenitor escapes normal proliferation control is where an autocrine loop develops with the secretion of a growth factor which can bind to a receptor which the cell already possesses. This was seen in transgenic studies, where retroviral expression of IL-3 or GM-CSF in transgenic mice resulted in non-leukaemic myeloproliferation (58;59). IL-3 has also been found to be occasionally over-expressed as a result of a translocation in a human acute pre-B cell leukaemia(60). These transgenic studies suggest that other genetic anomalies (possibly secondary to the proliferation following up-regulation of IL-3) are present and required for the leukaemia to develop. Co-transfection of a homeobox protein Hox-2.4 with IL-3 now results no longer in a myeloproliferative disorder but in myeloid leukaemia (61) probably by causing a block in differentiation.

Another mechanism for bypassing proliferation control is by activating the cellular pathway driving proliferation at a point further downstream. This can happen by means of a mutation resulting in a constitutively active surface receptor or signal transduction kinase (an enzyme in the pathway which normally carries the signal from a surface receptor towards the nucleus) resulting in proliferation. Such a mechanism occurs in the chronic phase of chronic myeloid leukaemia - which is basically a myeloproliferative syndrome with excessive amounts of white blood cells, which however differentiate normally. The Philadelphia chromosome-associated translocation results in the formation of a constitutively active tyrosine kinase gene *bcr-abl*, which drives this myeloproliferation (62). Complementation of this proliferation by a differentiation-blocking molecule such as the AML1/EVI-1 fusion protein (identified in the blast crisis of CML) results in an acute leukaemia (63).

Myelodysplastic syndromes (MDS) which often result in low numbers of differentiated peripheral blood cells but normal or slightly increased marrow progenitors due to an apparent block in differentiation can also be complemented by mechanisms increasing their proliferative potential, resulting in leukaemia. In a set of patients with MDS who were being given growth factor supplementation in order to help increase their peripheral blood counts, a bone marrow picture with features of acute leukaemia developed in certain patients. This resolved once these factors were withdrawn (64). Whether the spontaneous clinically recognised transition of myelodysplastic syndromes into leukaemias follows a similar or totally different mechanistic pathway, the conversion to leukaemia will usually be associated with an increase in cellularity. This would be likely to result from a complementary proliferative signal originating in one of the maturation arrested myelodysplastic cells.

1.2.2 Mechanisms of leukaemogenesis

Various agents may cause the combination of maturation arrest and proliferative disorder resulting in leukaemia. Intrinsic (genetic) defects within the leukaemic clone are passed down to the daughter cells of the transformed founder cell. Certain recognised causative factors such as irradiation and certain carcinogenic chemicals (including chemotherapy for other malignant diseases) cause leukaemia as a result of genetic damage, which they bring about in DNA of haematopoietic precursor cells (65).

Apart from genetic anomalies, the most common and apparent of which are chromosomal translocations, viruses are well known to play a strong etiological role in leukaemogenesis though this has been seen more often in animals than in man. Avian

myeloblastosis and myelocytosis viruses are well known to cause acute myeloblastic and myelocytic leukaemia respectively in chickens. In these cases the *v-myb* and *v-myc* oncogenes respectively may simultaneously block differentiation and stimulate proliferation (66;67). The Moloney murine leukaemia virus (68) and the spleen focus forming virus (SFFV) both cause leukaemia secondary to retroviral integration. In the latter case, though the virus contains no obvious oncogene, erythroleukaemia is induced due to the *env* gene protein of the virus, binding to and activating the erythropoietin receptor - a case of molecular mimicry. This makes the cell, growth factor independent (69) (and other changes probably secondary to this proliferation cause development of the leukaemia). Simultaneously, viral integration positions a viral long terminal repeat (LTR) next to a gene for a transcription factor (PU.1) which is thought to be responsible for its over-expression (70). This over-expression contributes to leukaemogenesis by blocking differentiation (in the case of erythroid activation of PU.1).

In humans viruses thought to play a role in leukaemia include the human T-cell lymphotropic virus-1 (HTLV-1) which is a necessary event in the development of a specific type of adult T-cell leukaemia and the Ebstein Barr herpes virus which is responsible for Burkitt's lymphoma as well as possibly other illnesses (71).

1.2.3 Classification of Leukaemia

Leukaemias have classically been subdivided into acute and chronic depending on the rapidity of progression of the disease and as myelocytic or lymphocytic according to the lineage characteristics of the predominant cells seen in Romanowsky-stained bone marrow samples (72). This results in four major subgroups of leukaemia namely acute myelocytic leukaemia (AML), acute lymphocytic leukaemia (ALL),

chronic myelocytic leukaemia (CML) and chronic lymphocytic leukaemia (CLL). Whilst a definitive classification of these malignancies is well beyond the scope of this introduction, a brief outline of classical classification follows.

Lymphocytic leukaemias are subdivided into those of T-cell or B-cell origin though these subsets may be further subdivided according to the stage of development at which lymphocyte development is arrested such as B, pre-B or pro-B (73). Some particular variants are separately classified according to a particular morphology of the abnormal leukocyte - such as hairy cell leukaemia (74). Chronic myelocytic leukaemia is more simply divided into Philadelphia chromosome positive or negative disease (75). AML is a more heterogeneous disease and has been generally classified by means of the predominant cell type's morphology as shown in the widely used French American British (FAB) classification (Table 1).

Lymphomas are also malignant haematological disorders, being lymphoid proliferative diseases more usually localised to solid lymphoid tissues. They have historically been classified as Hodgkin's disease and non-Hodgkin's lymphomas (a large group, including some variants of chronic lymphocytic leukaemia). Another lymphoid malignancy is multiple myeloma where the predominant cell type is a plasma cell - the end stage functional cell of the B-lymphocytic lineage (76).

Cytogenetic and molecular tools are nowadays helping dissect the aetiology of these and other diseases more clearly. Discovered genetic abnormalities have also thrown new light on to the biology of various leukaemias, are helping more specific diagnosis and have begun to influence management (77).

FAB Classification	Common terminology	%
M0	Acute myeloblastic leukaemia (without cytologic maturation)	0 (below)
M1	Acute myeloblastic leukaemia (with minimal maturation)	20(with M0)
M2	Acute myeloblastic leukaemia (with maturation)	30
M3	Acute promyelocytic leukaemia	10
M4	Acute myelomonocytic leukaemia	25
M5	Acute monocytic leukaemia	10
M6	Acute erythroleukaemia	4
M7	Acute megakaryoblastic leukaemia	1

Table 1. The French American British (FAB) classification of Acute Meloid Leukaemias. Some of the subtypes can be further subdivided. For example, M3 also includes an unusual hypogranular form whilst M4 can also have specific eosinophilic or basophilic features. Other rare varieties which do not fit neatly into any group include acute mixed lineage leukaemias which also include lymphocytic markers, and undifferentiated leukaemias where the malignant clone has a progenitor cell phenotype.

1.2.4 Genetic abnormalities in leukaemia

Genetic abnormalities are common in leukaemia; in fact clonal chromosomal abnormalities are found in 80% to 90% of children with acute leukaemia (78). The most common anomalies are balanced chromosomal translocations. They have been shown to play an important causal role in leukaemogenesis in certain cases, as in the case of the BCR-ABL fusion protein in CML and the PML-RARA fusion protein in acute promyelocytic leukaemia (79;80). 25% of childhood cases of ALL and 50% of cases of AML are related to phenotype-specific balanced translocations with characteristic biological and clinical features (78). A list of the commoner known chromosomal anomalies found in acute childhood leukaemias, with the associated molecular defect resulting from this translocation, is shown in Table 2. The gene products of such chromosomal translocations may be fusion proteins, which act as dominant negative transcription factors (as appears to be the case with PML-RARA and AML/EVI-1), which block normal differentiation (63;81). Other translocations disrupt apparent tumour suppresser genes as seems to be the case with the TEL/AML1 fusion protein, which is very often associated with loss of the other TEL allele (82). In certain lymphomas, other translocations result in over expression of cellular oncogenes like myc (83).

Common cytogenetic anomalies in adult leukaemias include the Philadelphia chromosome t(9;22)(q34;q11) found in over 90% of adult chronic myeloid leukaemias (75). Chromosomal anomalies are also detected in 50-65% of patients with chronic lymphocytic leukaemia. The most common of these are trisomy 12 and abnormalities of the long arm of chromosome13 (76).

Leukaemia type	Cytogenetic anomalies	Genes involved	Frequency (as % in ALL or AML overall)
B-lineage ALL			
	t(12;21)(p12-13;q22)	TEL-AML	22(molecular analysis)
	t(1; 19)(q23;p13.3)	E2A-PBX1	6
	t(9;22)(q34;q11)	BCR-ABL	3
	t(4;11)(q21;q23)	MLL-AF4	2
B-cell ALL			
	t(8;14)(q24;q32)	MYC(IGH)	1-2
T-cell ALL			
	-	TAL	4
	t(11; 14)(p13;q11)	TTG2(TCRD)	1
AML			
	inv(16)(p13q22)	CBFβ-MYH11	8-10
	t(8;21)(q22;q22)	AML1-ETO	8-10
	t(15;17)(q21;q21)	PML-RARA	6-10
	t(9;11)(p21-22;q23)	MLL-AF9	7-9
	t(1;22)(p13;q13)	unknown	2-3
	t(9;22)(q34;q11)	BCR-ABL	<1
	inv(3)(q21q26)	EVI1	<1

Table 2. Commoner known translocations and associated genetic rearrangements found in acute childhood leukaemias. The sign "-" indicates that no gross chromosomal anomaly is detected.

1.2.5 Clinical significance of leukaemia biology

The cytogenetics of leukaemia is now recognised as an essential prognostic factor in assessing the severity of disease and also in certain cases, in directing appropriate treatment to the condition. Acute promyelocytic leukaemia (APL), which is almost always characterised by a t(15;17)(q22;q12-21) balanced translocation, is a case in point. The translocation in this leukaemia results in the formation of the abnormal transcription factor RARα-PML. Differentiation therapy for this form of leukaemia with all-*trans* retinoic acid (ATRA) counters the inhibitory dominant negative effect of the fusion protein. This allows the leukaemic cells to differentiate to near-normal granulocytes which then apoptose normally (84). This has resulted in markedly improved remission rates and when combined with low dose chemotherapy, has very significantly improved cure rates, without developing the marrow hypoplasia common with usual modalities of treatment (chemotherapy). This first instance of successful differentiation therapy was specific to the abnormal transcription factor since a rare subtype of APL with a differing translocation producing the PLZF-RARα fusion protein did not respond similarly (85). It suggests a new way forward in the treatment of these diseases based on a greater knowledge of the causative anomalies in each particular instance. Even when using more standard modalities of treatment, cytogenetic and molecular abnormalities are now being more recognised as clear markers of prognosis.

IN AML, for example, patients with t(8;21) and inv(16) had the longest complete remissions and the best overall survivals whilst the worst prognosis was seen in patients with del(5q)/5q-, del (7q)/7q-, t(9;22) and rearrangements involving 11q23

except for the t(9;11) rearrangement (86). Prognosis for patients with leukaemia will presumably continue to improve in future due to treatment modalities tailored more specifically to the underlying molecular defect.

1.3 Models for studying haematopoiesis and leukaemia

In order to study haematopoiesis and leukaemogenesis in an experimental setting, various model systems have been developed over the years. Each method is more useful for investigating one or another aspect of blood cell biology and replicates the conditions of proliferation and differentiation in the bone marrow to a greater or lesser extent.

1.3.1 *In vivo* model systems

Haematopoietic cells have been long since studied by transferring either unfractionated bone marrow or cells with certain characteristics (such as CD34+ve cells, which would include the stem cell population,) into lethally irradiated mice. By seeing how much of the normal pattern of haematopoiesis is recovered by the transplanted cells, knowledge of the proliferative capacity and differentiation characteristics of transplanted cell population is obtained (87).

Whilst the above system was useful in studying haematopoiesis in the mouse, human haemato-and leukaemo-poiesis could be similarly studied *in vivo* only after the identification of the SCID (severe combined immunodeficient) mouse. This mouse carries a genetic defect, which results in it being incapable of mounting either a

humoral or cellular immune response. It can therefore be transplanted with autologous grafts or even xenografts from normal or leukaemic human bone marrow, whereupon such tissue continues to develop within the mouse and can be studied in this environment (88).

More specific questions into the function of certain genes in haematopoiesis have been addressed *in vivo* by means of transgenic or knockout mice, where a particular gene is added to, or deleted from, the mouse genome respectively (89). Whilst such experimental strategies may clearly indicate the importance of certain factors for particular aspects of haematopoiesis, they lack the flexibility to study the role of a particular gene product with time, or in relation to other factors or in different conditions. For more interventional experimentation, *in vitro* model systems come into their own.

1.3.2 *In vitro* model systems

The long term culture of bone marrow and peripheral blood stem cells *ex vivo* has improved greatly in the last decades greatly due to the recognition of the interaction between haematopoietic cells and their microenvironment, particularly stromal cells in the bone marrow (90). Originally by growing blood stem cells in contact with stromal cell 'feeder' layers and more recently by direct supplementation of many of the purified cytokines and other growth factors provided by these stromal cells, haematopoiesis can be maintained over months in long term liquid cultures (5). The continuing access to the cells allows manipulation of growth conditions over time and provides a flexibility lacking in *in vivo* models for studying differentiation-related decisions taken in blood progenitors over time and/or under different conditions.

1.3.3 Myeloid cell lines as models of myelopoiesis

Both sets of experimental models described above are complex systems requiring considerable care and attention but may provide the most accurate information into the actual process of haematopoiesis. The use of leukaemic cell lines (which are often growth factor independent) to study more specific aspects of blood cell biology is a convenient and established method, provided adequate care is taken in the interpretation of results. Thus the K562 cell line (erythroid blast crisis of CML) has often been used in the study of foetal haemoglobin expression since this cell line is capable of expressing foetal globins to a high level (91). Likewise many myeloid cell lines such as HL60 (a myeloblastic cell line with the bi-directional differentiation capability of a promyelocyte) and U937, (a monocytoid derivative of a histiocytic/lymphocytic cell line) are often used to study the regulation of genes expressed at different time points in myeloid maturation (92). According to their particular phenotypes, each cell line is taken to represent the myeloid precursor cell at the stage of differentiation similar to that at which the leukaemic clone is arrested. Therefore different cell lines can be taken to represent different time points in the myeloid differentiation pathway.

The leukaemic nature of these cells encourages caution due to the fact that differences between the cell line and untransformed primary cells are certain (such as the lack of expression of most secondary granule proteins in differentiated myeloid leukaemic cells) (92;93). The easy transportation and maintenance of cell lines (as opposed to primary tissue) as well there relative conformity and the abundance of material reduces variability that would occur due to many different patient sources. This

allows scientists in different laboratories to collaborate more closely and minimises difficulties in interpretation due to variant test conditions.

1.3.4 NB4 as a model of neutrophilic and monocytic differentiation

NB4 is a cell line derived from a relapsed case of acute promyelocytic leukaemia (APL), which as the name implies is a leukaemia where cells are arrested at the promyelocyte stage of differentiation (94). Promyelocytic leukaemia has been treated recently by differentiation therapy as described above but this treatment requires the presence of a t(15;17) translocation resulting in the formation of the hybrid transcription factor PML-RARA. The NB4 cell line, being derived from such a leukaemia with the characteristic cytogenetic abnormality maintains the capability to be differentiated to cells of near- normal neutrophil granulocyte morphology in the presence of all-*trans*-retinoic acid (ATRA). Further research using this cell line has shown it can also be induced to differentiate to monocytoid phagocytic cells using different combinations of inducers such as phorbol ester together with activated di-hydroxy vitamin D3 (95). Thus, despite a complex karyotype with hypotetraploid chromosome number in most metaphases, the normal pattern of bi-potential promyelocyte differentiation was relatively well preserved and this leukaemic cell line can be used as a model for myeloid differentiation. Particularly relevant to this study, NB4 cells had been shown to express defensin (96) so this cell line could be used to study the regulation of *defensin* gene transcription.

Chapter 2 : Transcriptional regulation of gene expression

Gene expression, which is largely responsible for the particular phenotype of cells and indirectly of tissues and organisms, is controlled in many different ways. Much control is exerted at the initial stage of transcription where messenger RNA (mRNA) is produced from the genomic DNA template. Processing of the mRNA into a mature form, through splicing, capping and polyadenylation, translation of the mRNA into protein and subsequent post-translational modifications are subsequent stages of the process of conversion of a coded message within the DNA blueprint into a mature functional protein. The presence or absence of these proteins is what eventually determines a cell's structure and function. Each of these steps in protein production may be regulated thereby allowing for a very fine control of gene expression to be achieved.

2.1 A general overview of the elements of transcriptional control

The transcription of a gene depends on *cis*-acting DNA elements upstream (and/or downstream) of the coding region as well as on *trans*-acting factors which interact with these elements. *Cis*-acting elements may be positioned just upstream of the gene in question (usually referred to as promoter elements) or may be situated more distantly both up- and down-stream of the gene (enhancer elements). Transcription can be divided into a number of individual steps, which occurs in a stepwise fashion. These are pre-initiation complex (PIC) assembly, DNA melting (PIC activation), transcription initiation, promoter clearance, elongation and termination. Binding of

transcriptional proteins to the core promoter elements (as part of the pre-initiation complex) has been shown to be a major rate-limiting step for the transcription of most promoters *in vitro* (97;98). In some promoters, however, an initiation complex seems to be stably bound to the DNA and in these cases , it is the later steps of transcription, which are likely to be responsible for regulation (99). All these interactions, though easily identified in *in vitro* experiments, are dependent, *in vivo*, on the chromatin packaging of the promoter DNA.

2.2 Chromatin and Transcription

Chromatin can be defined as the genomic DNA together with the associated proteins, including both structural and regulatory molecules. Much work in recent years has indicated that the process of transcription is intricately interlinked with the regulation of chromatin structure by chromatin-modifying molecular machinery - reviewed in (100).

2.2.1 Nucleosomes

The basic unit of chromatin is the nucleosome. About 150 bases of DNA are wound 1.8 times around a nucleosome core made of two molecules each of histones 2A, 2B, 3 and 4(101). Histone 1 (often called a linker histone) binds to the DNA at either end of the nucleosome and encourages chromatin condensation. The primary chromatin fibre of genomic DNA wrapped around nucleosomes is once again coiled upon itself to form a 30nm fibre, which forms the bulk of interphase chromatin. Recent studies have

shown that the RNA polymerase unwraps DNA from the nucleosomes as it transcribes along the DNA template so that the nucleosome is displaced to the DNA behind the polymerase without the latter ever actually loosing contact with the DNA(102). Despite being able to read through nucleosomes uninterrupted, considerable pausing occurs during the process, the overcoming of which is crucial, to allow adequate transcription of a chromatin-embedded gene.

2.2.2 Silent and active chromatin

Chromatin has long been divided rather archaically into denser heterochromatin and euchromatin. Actively transcribed genes are usually found within euchromatin (103). Genes within heterochromatin are almost always repressed as is clearly seen in X-chromosome inactivation (104).

The effects of chromatin on gene expression are most clearly seen in stable transfection experiments and related transgenic experiments where the expression of a gene depends greatly on where in the genome it has integrated. Genes integrated close to the telomeric ends of chromosomes, for example are much more likely to be repressed. Introduced genes, which integrate adjacent to a region of heterochromatin (though this could be hundreds of kilobases away), undergo the phenomenon of positional effect variegation. These genes are occasionally expressed and occasionally not resulting in variable phenotypes within different transgenic daughter cells (105). The exact explanation for this phenomenon is unclear. Such repression (whether stable or variegated) differs considerably from the situation in transient transfections where promoter activity is determined primarily by the absence/presence of the appropriate

transcription factors in the cellular environment and their binding sites within the promoter sequence.

2.2.3 Enzymatic modification of chromatin

The enzymatic acetylation of histones plays an important role in transcriptional activation of chromatin-bound templates and is discussed further in section 2.4.8. Other enzymes deacetylate histones and therefore return nucleosomes to a more stably DNA-bound state. These latter enzymes appear to repress transcription (106). Other enzymes methylate DNA, thereby inhibiting the binding of certain transcription factors to their binding sites and encouraging the binding of other methylation dependent DNA-binding proteins. This methylation of DNA also represses transcription (107). As well as these specific modifications, a large number of enzymes and protein complexes including the SWI/SNF complex (100) have been identified to play an important role in the modification of chromatin. Such complexes usually include proteins with acetylase and deacetylase activity as well as molecules, which appear to be ATP driven molecular motors, which shuttle along the DNA. Though the exact mechanism of action of these various complexes is as yet unclear, the activities of the chromatin modifying machinery impinge on transcription and provide yet another level of control into this process which defines the characteristics of living cells.

2.3 Sequences involved in the regulation of transcription

2.3.1 Promoters and initiators

Core promoter elements are defined as the 'minimal DNA elements that are necessary and sufficient for accurate transcription initiation by RNA polymerase II in reconstituted cell-free systems' (108). The most well known such elements include the TATA box which is usually located 25 to 30 bases upstream from the transcriptional start site, and the initiator; a pyrimidine rich sequence (consensus YYAN$^{T}/_{A}$YY) located at the transcriptional start. Promoter is a rather loosely used term but it generally refers to the sequence of the gene directly upstream from the transcribed sequence which is required for strong expression of the gene (or a substituted reporter gene) in its normal cellular environment. Whilst a promoter is often tissue-specific, core promoter elements are not (108).

2.3.2 Modularity of promoters

Gene promoters are made up of different recognised modules (short recognised sequences) which are required in order to allow the promoter to function. These modules may be spread out over 100 bases or more. These usually include one or more core promoter elements, found in many different gene promoters, as well as other activator binding sequences, which whilst not necessary for accurate transcript initiation are nonetheless required for high level gene/reporter transcription, such as CAAT boxes (109). Such modularity of promoters and also of enhancers (see below) allows the

building up of different combinatorial *cis*-acting factor complexes using binding sites for a limited number of transcription factors. Adjacent to and often overlapping with promoter sequences, upstream of genes, are sequences rich in G and C nucleotides. These GC-rich regions are a target for methylation, which by reducing the binding of methylation-sensitive activator proteins, can inhibit the expression of the adjacent genes (107).

2.3.3 Enhancers

Enhancers as their name implies, are sequences which enhance transcription initiation (110). They are capably of mediating this effect at a distance from the site of transcription initiation, and independent of their orientation. They may be found both 5' and 3' to the transcribed gene sequences. They are often tissue specific. Whilst they are also modular, the different activator-binding consensus sequences are more often contiguous unlike in promoters. They can usually enhance the activity of diverse promoters placed adjacent to them rather indiscriminately by allowing activator binding in their proximity (111).

2.3.4 Locus control regions

Also referred to as dominant control regions and bearing certain functional similarities to the long terminal repeats in retrovirus genomes, these LCRs are exemplified by the cluster of super hypersensitive sites (in erythroid cells) 10 kb

upstream of the β-globin cluster. Adding these sequences to a β-cluster globin gene with its promoter before introduction into a mouse (thereby creating a transgenic), resulted in high level tissue-specific expression similar in extent to the expression of the endogenous mouse gene and in direct relation to the copy number of integrated genes (112).

Whilst many of these features are also features of strong enhancers, the LCR allowed this high level expression independent of the position of integration in the mouse genome (without the LCR, transgenic genes are usually very poorly expressed and then this depends on the site of integration). One element of the LCR, (hypersensitive site 2), whilst still capable of causing high level position independent expression in stably transfected transgenes or in transgenic mice, was incapable of enhancing linked globin gene transcription in transient transfection assays. This indicates that this sequence is specifically involved in chromatin-dependent regulation of transcription but is not a classical enhancer thus allowing distinction between the two types of regulatory DNA sequences.

2.4 Proteins regulating transcription

2.4.1 RNA polymerase II

All nuclear genes are transcribed from DNA by one of three RNA polymerases. Polymerase I exclusively transcribes the 5.8S, 18S and 28S ribosomal RNAs.

Polymerase II transcribes genes encoding messenger RNA and certain specific small nuclear RNAs. Polymerase III transcribes t RNA genes, the 5S rRNA and other small nuclear ribosomal RNAs (113).

Some studies suggest that the RNA polymerase II is bound to DNA as part of a stepwise process following the previous binding of DNA-binding general transcription factors (114). However some recent work suggests that the polymerase may exist as a pre-formed complex together with various general transcription factors and co-activators known collectively as the RNA polymerase II holoenzyme (115). This complex may then be recruited as a single unit by means of interactions with DNA-bound activators and core-binding factors. The stepwise process of basal transcription machinery recruitment is described below.

2.4.2 General transcription factors

These proteins together with RNA polymerase II comprise the basal transcription apparatus, which recognises the core promoter and initiates transcription - reviewed in (108). They are the TATA-binding protein (TBP) and transcription factors (TFs) TFIIB, TFIIE, TFIIF and TFIIH. These sub-units are conserved between all eukaryotic species, even in yeast. TBP recognises known core promoter elements. TFIIB binds to the promoter by means of interactions both with TBP and directly with DNA adjacent to this molecule. It then recruits an RNA polymerase-TFIIF pre-formed complex by means of interactions with both proteins. TFIIE is recruited to the complex via interaction with the polymerase, upon which it then recruits TFIIH, a factor with helicase, ATPase and kinase activity, which melts the promoter DNA (116).

Transcription of the gene DNA can then begin whilst phosphorylation of the polymerase C-terminal domain probably results in destabilisation of interactions with other promoter bound factors such that promoter clearance and elongation can proceed (117).

2.4.3 TBP and the TBP-associated factors (TAFs)

TBP is normally found in association with various factors (TAFs) making up a complex known as TFIID (118). TBP and certain other TAF components of TFIID are the only general transcription factors (GTFs) known to bind directly to DNA. There is clear evidence even *in vivo* that their binding to the promoter is a major rate-limiting step in transcription (119). The TAFs are likely to be important in targeting TFIID to promoter elements which lack a canonical TATA sequence and which therefore do not strongly bind TBP. Such promoter interactions may also require other factors like TFIIA which seems to be essential in stabilising promoter-TFIID interactions in the absence of a TATA box, probably by interactions with the TAFs (120).

TAFs also play an essential role in activator-enhanced transcription. This was recognised since TBP alone could substitute adequately for the whole TFIID complex as regards basal transcription, in a cell-free test system with purified proteins. However activator-enhanced transcription was seen to require TAFs (121). Interestingly it was noted that different classes of activation domains bound to different TAFs - reviewed in (118). Transcription factors Sp1 and *Drosophila* TF bicoid (which have glutamine rich activation domains) bind to *Drosophila* TAF_{II} 110, whilst p53 and the viral activator VP16 (acidic activation domains) bind to TAF_{II} 40. By reconstituting TFIID from its constituent sub-units, it was shown that transactivator enhanced transcription was

dependent on the presence of the TAF known to interact with that particular transactivator (122).

Thus transactivators may enhance transcription by recruiting the basal apparatus to a promoter more efficiently (123) probably via their interactions with TAFs . This appears to be due to more (as opposed to faster) transcription complex formation on the promoter (124). Various other factors, which are not so tightly bound to the basal transcription machinery as the TAFs, are also required for activator-mediated enhancing of transcription. These are referred to as co-activators and will be discussed (see section 2.4.8). The interactions between all these different classes of factors are represented in Fig.4.

2.4.4 Activators

Many transcription factors, including c-Fos, c-Jun, c-Ets and others often act as activators and are distinct from the factors involved with the basal transcription machinery. Activators are defined as such, depending on their capability to transactivate a promoter - *in situ* within the genome or more commonly linked to a reporter gene (125). In most cases this is dependent on the activator binding a site within the promoter or a linked enhancer sequence. As described above, activators interact with the basal transcription machinery by means of the TAFs, thereby helping either recruit the basal machinery, or stabilise complex assembly on the promoter thereby increasing the rate of transcription by enhancing initiation. In order to do this, most activators therefore require a DNA binding domain to bind to their characteristic recognition sites (cis-DNA-elements) within a promoter or within enhancers, as well as

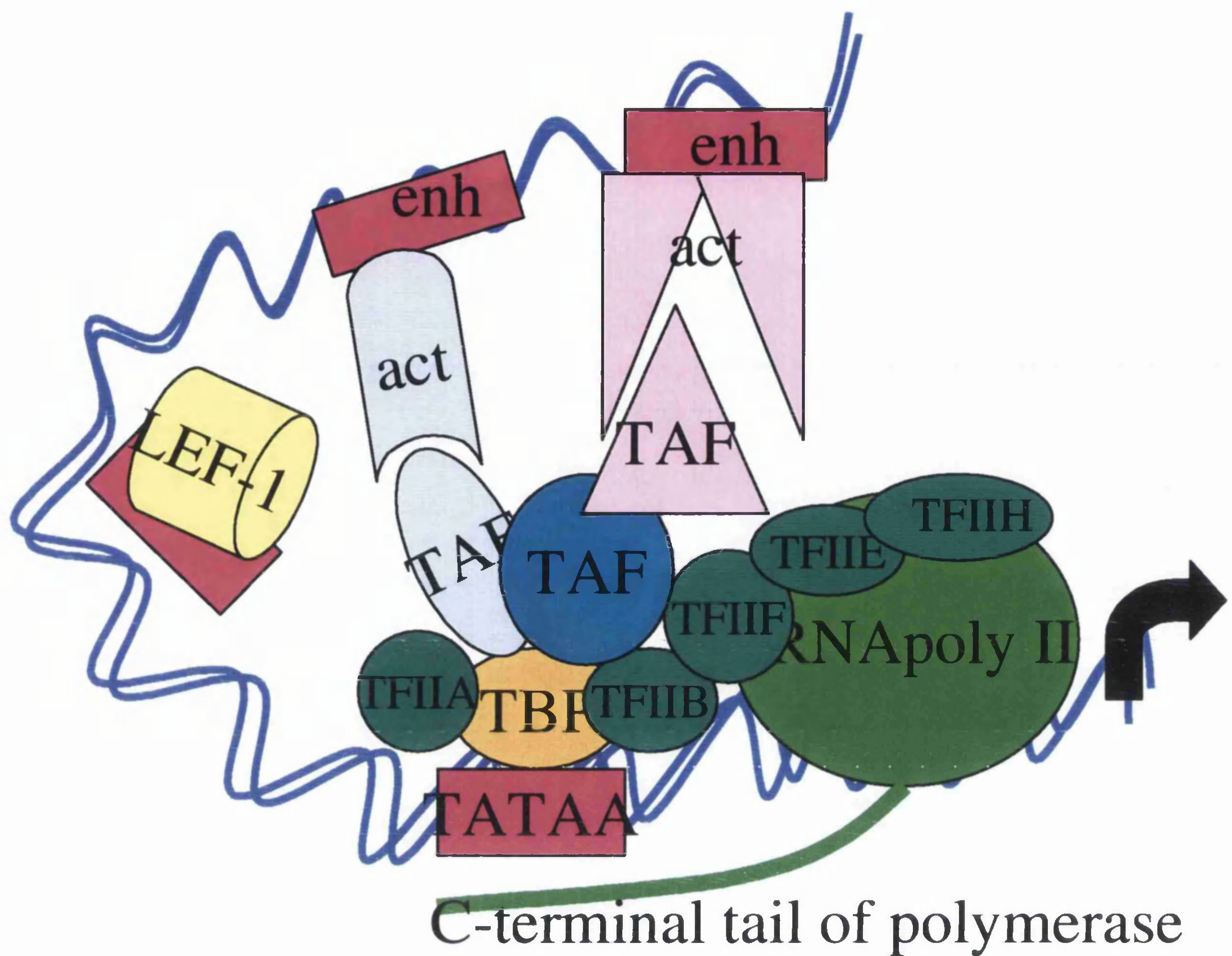


Figure 4. Anatomy of a transcription factor complex. The interactions of various activators (act) bound to enhancer sequences (enh) with different TBP-associated factors (TAFs) help recruit TBP to the TATAA box. Then through interactions between TBP and the other general transcription factors TFIIB,F,E,H, etc, the RNA II polymerase is itself recruited to the promoter and initiation of transcription can begin.
LEF-1 is included as an example of a co-activator which whilst not itself directly involved in recruiting the polymerase, still performs an essential function. It kinks the DNA (shown here in blue) thereby allowing the enhancer sequence-bound activators to interact with TBP thereby stabilising the pre-initiation complex on the TATAA box.

an activation domain by means of which they can interact with the transcription machinery. Some activators are incapable of binding DNA as monomers and therefore need to form dimers or multimers with themselves or with other transcription factors in order to establish the link between the cis-elements with which they interact and the basal transcription machinery. c-Jun and c-Fos, for example, interact by means of a common protein-protein interaction domain, the leucine zipper, to form a heterodimer, the transactivator AP-1 (126). GABPβ, another transactivator which has a strong transactivation domain but no DNA binding domain, is attached to the DNA by means of a protein-protein interaction with a DNA-binding factor GABPα which has little inherent transactivation activity due to the apparent lack of a strong transactivation domain (127).

Thus domains commonly found in *trans*-activators include a DNA-binding domain and a transactivation domain which interacts with the proteins of the basal transcription machinery. Protein interaction domains allow for binding to other activators and co-activators whilst nuclear localisation sequences (which may not be a separate domain) allows the protein to be localised in the nucleus once it has been translated in the cytoplasm (128). Transactivators may also have other domains with specific functions such as ligand-binding in the case of the steroid receptors (129).

2.4.5 DNA-binding domains

A number of different protein domains allow transcription factors to interact with either the major or minor grooves of the DNA double helix structure. Many such domains are basic (net positive charge) in nature, allowing them to interact with the

negatively charged phosphate backbone of the DNA. The different DNA-binding domains are classified according to the similarity of the domain to that of previously identified factors (e.g. ETS domain, MYB domain etc). Otherwise, they may be grouped according to particular structural features of the domain (e.g. zinc finger domain; a finger-like motif held in shape by interactions of amino acids within the peptide chain with a central zinc atom). Different types of DNA-binding domains found in different transcription factors are shown in Table 3 (130).

2.4.6 Transactivation domains

Different transcription factors also interact with the basal machinery by means of different transactivation domains. As has been described above, the different classes of activation domains may interact with the basal machinery through different TAFs within TFIID. Types of transactivation domains include acidic domains, as found in the tumour suppresser gene p53 (131), glutamine-rich domains, as seen in GABPβ (132)and Sp1 (133) and proline-rich domains as found in the CAAT transcription factor CTF (134). Some transactivators have more than one type of transactivation domains such as PU.1 which sports both acidic and glutamine-rich domains(135).

2.4.7 Protein interaction domains

As described above, many transcription factors interact with other factors in order to bind DNA, both due to a complete lack of an endogenous DNA-binding

DNA-binding domain	Transcription factors binding DNA through this type of domain
Helix-turn-helix (HTH)	Myb, Hepatic Nuclear Factor 1(HNF-1), Oct-1,Ets (in this case the domain is a winged HTH)
Homeodomain - contains an HTH motif	Antennapedia (*Drosophila*), Engrailed (*Drosophila*), HoxA, HoxB
Helix-loop-helix (HLH)	MyoD, E2A
Basic zipper (b/Zip) – contains an HLH motif and a leucine zipper	Myc, Max, Jun, Fos, C/EBP, CREB
Zinc finger domain	MZF-1, GATA-1, Evi-1, ZEB
Steroid receptors – may include one or more zinc finger-type structures	Estrogen receptor, Glucocorticoid receptor
Other diverse	P53, High mobility Group proteins, AML1, NFκB

Table 3. Transcription factor DNA-binding domains.

As can be seen there is a considerable overlap between various domain structures. Some transcription factors may also contain more than one different kind of DNA-binding domain as do the POU proteins which have both a homeodomain and a separate HTH domain. Some of the structures involved are only indirectly involved with DNA binding as in the case of the leucine zipper within the basic zipper proteins. It is the basic region which is actually responsible for DNA interaction but the leucine zipper is essential in mediating protein-protein interactions. Protein heterodimers formed through interactions via this domain are then capable of binding DNA more strongly and specifically.

domain (as in GABPβ) or due to an improved DNA binding and cis-element targeting (as in the C/EBPs or c-Fos and c-Jun). Such interaction domains again differ in various transactivators (136). In many cases they involve one or more alpha helices in a variety of configurations. One of the most common protein interaction domains is the leucine zipper, found in the C/EBPs, an alpha helix with a periodic arrangement of leucine residues (137). These residues allow two such domains on C/EBP monomers to interact hydrophobically with each other, resulting in dimer formation.

2.4.8 Histone Acetyltransferases and other co-activators

As well as the basal machinery and the transactivators, a number of other factors also play a part in transcription (138). These are not tightly bound to the basal machinery as are the TAFs and are not themselves capable of transactivating of promoters. They do however enhance the activity of transcription factors, which are known to target a particular promoter. One hypothesis of how these factors work is that they act as bridges between transactivators and the basal machinery components (139). One such co-activator, the CREB binding protein (CBP) and the very similar p300 protein, have both been shown to interact with multiple transactivators thus supporting this possible mechanism (140). These two proteins as well as a number of other co-activators have a certain degree of histone acetyltransferase (HAT) activity(141).

Acetylation of histones, particularly the N-terminal tails of H3 and H4 histones, reduces the stability of histone-DNA binding (142). Thus apart from playing a (bridging) role in pre-initiation complex formation, certain co-activators with HAT activity, may also be involved in opening up the chromatin template and allowing

improved access of other transactivators thus further improving recruitment of the basal machinery of transcription.

2.5 Regulation of Transcription factor activity

Transcription factors can be positively or negatively regulated by a number of different processes - reviewed in (143). The rate of transcription and translation of the factors themselves may be regulated, thereby effecting the expression of their downstream target genes

As the only mechanism of gene regulation, however, this would result in an increasing pool of upstream factors regulating more downstream factors *ad infinitum.* Eukaryotic organisms reduce the number of factors required for gene control by having modular promoters. The binding of more than one transcription factor in different combinations to these promoters, according to the binding sites (modules) present, can differentially regulate various genes. Multiple forms of some transcription factors produced either by splicing variation or by translating the same mRNA using different start codons, introduces another level of control where the different variants of the same factor may have different transcriptional activity.

Post-translational modifications to these transcription factors and interactions between different factors to form various transcription factor complexes, allows a limited number of initial factors to regulate a large number of downstream genes in different circumstances and tissues.

2.5.1 RNA splicing and translation

A large number of genes may be regulated by the formation of differently spliced m-RNAs, which are then translated into proteins with different transactivation activities. One example is the myeloid transcription factor AML-1 (acute myeloid leukaemia-1). Numerous transcripts of this gene have been detected in normal blood cells probably as a result of the use of alternate promoters and polyadenylation sites as well as alternate splicing (144). One AML-1 transcript, termed AML-1A, lacks a transactivation domain and whilst it itself has no effect on the transcription of a target gene, it suppresses transcription by the longer splice variant AML-1B which has a transactivation domain. Over expression of the AML-1A splice variant suppresses the GM-CSF-induced differentiation of the 32D myeloid cell line, and enhances proliferation. Concomitant over-expression of the AML-1B bypasses this effect. It is not therefore surprising that many myelocytic leukaemia patients' cells express a relatively increased amount of AML-1A (144).

The transcriptional activity of both C/EBPα and C/EBPβ is regulated by the relative expression of two protein isoforms, a full length and an N-terminal truncated isoform; both translated from the same mRNA. The full-length isoform usually acts as a strong activator of transcription whilst the shorter isoform, being a much weaker activator, acts as a competitive repressor. Addition of serum as well as various hormonal stimuli such as insulin, result in alteration of the ratio between these two isoforms in liver/adipose cells, suggesting that they may be important in the metabolic and hormone dependent regulation of target genes (145).

2.5.2 Phosphorylation

Cell growth and differentiation signals are first recognised by cells at the cell surface following the binding of a growth factor/ligand or a cell surface molecule to a receptor on the target cell. This signalling results in activation (usually through the activity of a number of signal transducing G-proteins) of one or another kinase cascade like the MAP (mitogen activated protein) kinase cascade resulting in eventual phosphorylation of certain target transcription factors.

One such factor is c-Jun, which is phosphorylated on its transactivation domain by such kinases, resulting in an increase in transactivation activity (146). As a result of this, it can then trans-activate various target downstream promoters causing changes in gene expression. On the other hand, c-Jun's DNA binding is reduced due to phosphorylation by a constitutive kinase (147). A specific phosphatase dephosphorylates three different sites within the DNA binding domain resulting in increased DNA binding (146;147). Thus both phosphorylation and dephosphorylation can increase the ability of c-Jun to bind to and transactivate certain downstream promoters.

2.5.3 Proteolysis

A number of transcription factors are regulated by proteolysis. One of the most clinically relevant factors regulated by proteolysis is the product of the tumour suppresser gene p53. Ubiquitination of P53 and its targeting for proteolytic degradation follows the formation of a complex between P53 and the protein MDM2 (148). C/EBPβ can also be proteolytically degraded into a less active isoform in certain

circumstances and this degradation is apparently dependent on C/EBPα (149). This provides a medium whereby one C/EBP factor can reduce the competition by another C/EBP factor for the same DNA site. Proteolysis can be used to activate a transcription factor too. The inhibitory protein IκB is degraded by ubiquitination and proteolysis following phosphorylation (150) thus releasing the active transcription factor NFκB to which it is normally complexed in the cytoplasm (151). NFκB can then activate target genes, upon translocation into the nucleus.

2.5.4 Ligand binding

A number of transcription factors are regulated by ligand binding which converts an inactive factor into an active one. Foremost amongst these are the steroid, retinoid and thyroid receptors. All these transcription factors in the super family have a specific transactivation domain, and a ligand-binding domain, attached via a link sequence to a DNA-binding domain (129). In the case of the steroid receptors, hormone binding results in a configurational change, which results indirectly in the exposure of a ligand-binding/dimerisation surface through which homodimers of the ligand-bound receptor are formed. These dimers can then bind the palidromic hormone response element in DNA, and activate target genes.

2.5.5 Multimerisation

One very effective and widespread method which cells use to increase specificity of transcriptional regulation is the process of multimerisation. Different factors dimerise with other members of the same family and also sometimes with transcription factors of different families in order to produce specific complexes which can then activate diverse promoters according to the particular binding sites present. Such transcription factor complexes as well as differing in their ability to bind DNA, may also differ in their strength as transactivators. One of the most common dimerisation motifs is the leucine zipper, found in the C/EBP transcription factors as well as c-Jun and c-Fos, c-Myc and Max. A group of (usually) seven leucine residues present on one face of an alpha helix allows two proteins with this same motif to adhere via hydrophobic interactions between these leucine residues thus resulting in a dimer (137). In the case of Myc and Max, absence of the former results in the formation of a Max homodimer, which binds to promoters with the correct binding site (an E-box sequence) but is inactive (152). The increased presence of c-Myc results in a shift with the formation of more c-Myc/Max heterodimers, which now bind to the same site as an active complex.

Amongst the C/EBPs, dimerisation between different active forms can result in dimers of different specificity and activities (153), whilst dimerisation with other regulatory family members (e.g. CHOP-10), can result in dimers with defective DNA binding and thus reduced transactivation potential (154). The binding specificity of C/EBPs may also be modified by the interaction of monomeric forms with transcription factors of another family such as the ATF family of factors thereby targeting the newly formed complex to a different site (155).

2.5.6 Nuclear localisation

Most nuclear proteins have a primary sequence domain which directs their import into the nucleus (128). Transcription factors may be functionally active but kept inactive by compartmentalisation in a part of the cell where they cannot exert their effects. NFκB sequestered in the cytoplasm by IκB is one example. Degradation of IκB allows NFκB to be translocated to the nucleus where it is active (150). Steroid receptors also undergo a change in their affinity for the nucleus upon ligand binding probably secondary to the release of a large number of chaperone proteins, which are bound to the cytoplasmic steroid receptor. STATs (Signal transducers and activators of transcription) are an ever-growing family of cell surface molecules which translocate to the nucleus and activate transcription upon ligand binding(156). In *Drosophila*, the cytoplasmic membrane-bound protein Notch is proteolytically cleaved following ligand binding to its extra-cellular domain. Release of the intracellular portion of the receptor results in its nuclear translocation where it too activates target genes (157).

2.5.7 Transcription factor synergism and inhibition

As mentioned above, many transcription factors function optimally as part of dimers or multimers. Some multimers are made up of inactive sub-units, such as the Max homodimer. Other protein interactions, however, (such as that between C/EBPα and AML1) occurs between two transcription factors, each of which is capable of transactivating a test promoter (as assessed by reporter genes). On occasion, the effect of both factors on the promoter is considerably greater than the sum of the individual

effects of either factor suggesting that the increase is not due to two separate effects on the basal transcriptional machinery but a combined and somehow super-additive effect. This is the phenomenon of transcriptional synergism (158).

As well as collaborative effects, different transcription factors of the same or different families may inhibit each other's activity. This may be due to competition for the same DNA binding site, as probably occurs between inactive C/EBPγ dimers (159) and activatory C/EBP family members. It may also occur by formation of a complex incapable of binding DNA, as between the inhibitor CHOP-10 and other C/EBPs (154). Otherwise, the transactivation domain of a transcription factor may be specifically inhibited as occurs in P53 inhibition by MDM2 (160).

2.6 Cell/Tissue Specific Gene Transcription

All tissues and indeed even single cells of an organism (except for haploid gametes) contain the same genetic blue print within their DNA, the genotype. The phenotype of that tissue, its structural and functional identity, is however determined by which of those genes are expressed in its cells. This expression may be modified by surrounding microenvironmental stimuli coming from hormones, growth factors or from interactions with adjacent cells. During ontogeny, certain cells, on expressing a particular set of genes, take on a phenotype characteristic of the tissue involved, whilst other cells (also partly a result of their particular pattern of gene expression) die by apoptosis. The combination of these different outcomes results in tissue and organ formation in the embryo (161). Both cells which have yet to develop tissue-specific

characteristics (such as embryonic stem cells) and cells which have lost tissue-specific characteristics (such as tumour cells) may have different gene expression patterns, compared to those of differentiated tissue. Loss of expression of certain genes during tumorigenesis can be related to changes in morphology and in behaviour of the cells as can be seen with oestrogen receptor loss in breast cancer (162) and with loss of E-cadherin in prostate cancer (163). Occasionally tumours may become so genetically unstable that marked changes to the phenotype occur, with these tumours loosing the characteristics typical of their tissue of origin. When re-transfected with DNA from the parental tissue, this phenotype can be at least partially recovered (164). Amongst the genes which can cause such recovery are transcription factors which by regulating the expression of other tissue-specific genes can act as master controllers of phenotype (165).

2.7 Transcription factors and differentiation

Both *in vitro* experiments with certain particular transcription factors as well as *in vivo* experiments using transgenic animal and knockout strategies bear witness to the immense importance of transcription factors is tissue-specific development. Disruption of the C/EBPα gene in mice results in lack of early fat deposition even once the fatal hypoglycaemia is corrected (166). Knocking out the muscle-specific transcription factor Myogenin results in a major absence of skeletal muscle in new-born mice (167) whilst disruption of the Brn-3b transcription factor results in loss of a large majority of retinal ganglion cells (168). Whilst these studies indicate the importance of these transcription

factors in the genesis of particular tissues, the effects of such null mutants for particular genes are often less severe than expected. The apparent importance of C/EBPα in inducing an adipocyte phenotype from pre-adipocyte cells, as well as its role of up-regulating liver-specific gene transcription (169), would suggest a more severe phenotype upon disruption.

Similarly, disruption of the muscle specific master regulator MyoD, which can induce a virtually complete muscle phenotype when over expressed in certain heterologous cell lines (170), results in only a mild phenotype. Affected pups survive birth even though they are somewhat weaker than their normal siblings. This phenotype is less severe than expected probably due to an element of redundancy between different transcription factors of the same subgroup where one member may substitute functionally for another. Thus the function of C/EBPα in various tissues may be somewhat substituted for by other C/EBP members. In double knockout mice lacking MyoD and MRF4, (another muscle-specific bHLH transcription factor), new-born animals died from severe muscle defects unlike single knockout mice (171).

Whilst a single factor like MyoD can act as a master regulator and initiate a complete differentiation pathway in heterologous cells, this depends to some extent on the collaborating factors and inhibitory factors present in those cells. Thus, whilst an almost complete muscle phenotype was induced in 10T1/2 cells (170), other cells like HeLa cells did not show much muscle specific gene activation at all (172). Therefore whilst the importance of certain transcription factors in determining tissue identity is beyond doubt, both *in vivo* and *in vitro* experiments, indicate that different factors interplay with each other and with the general cellular environment, resulting in a considerably complex regulatory system determining cell and ultimately tissue fate.

The simple absence or presence of a transcription factor can influence cell fate determination, but it is the expression of a hierarchy of different transcription factors, throughout the process of differentiation of a precursor cell, which eventually results in a specific cell phenotype (165). Understanding such changing features is not so easy using the powerful *in vivo* techniques of transgenesis and knockout mice, but is more easily studied using *in vitro* experimental systems despite their obvious limitations.

Chapter 3 : Transcription factors in haematopoiesis

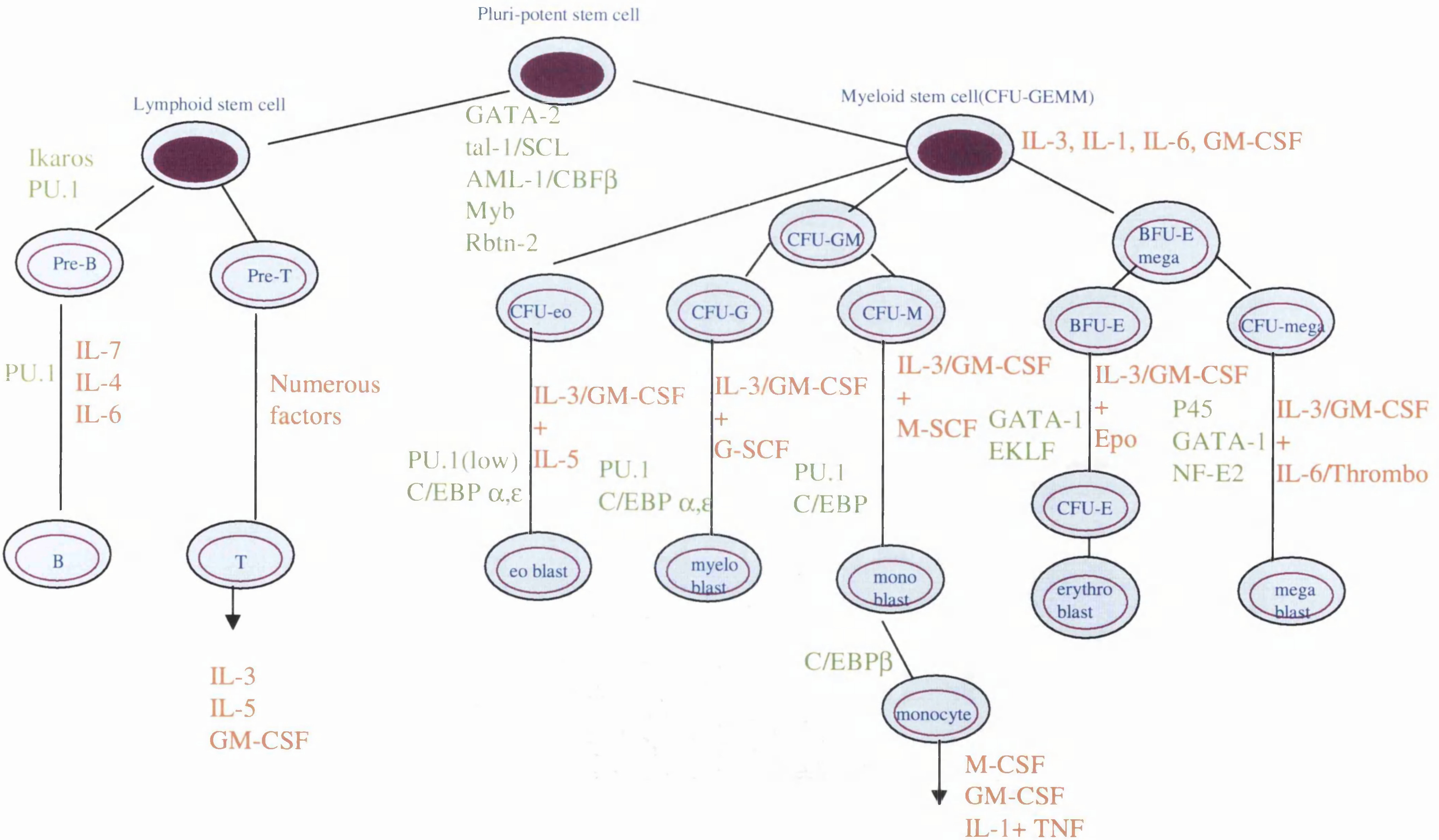
The process of myelopoiesis, and indeed all haematopoietic development, like the differentiation of any other tissue as outlined above, depends on the interaction of multiple transcription factors. Many haematopoietic transcription factors were originally identified due to their involvement in leukaemic translocations. Using techniques such as those described, of knocking out (disrupting) genes in mice as well as by expressing these transcription factors in different cell lines, their relative importance at different stages of differentiation can be assessed.

3.1 Transcription factors as effectors in haematopoiesis

Cell surface receptors, their ligands (including various cytokines and other growth factors) and transcription factors (ubiquitously expressed as well as tissue specific) are all required for haematopoietic development. It is, however, changes in the presence and concentrations of the latter, which are ultimately responsible for differentiation decisions. Their expression may be in turn regulated by signals coming from cell surface receptors, which also provide proliferative and survival signals to cells at various stages of differentiation. A model for such a mechanism of differentiation control is shown (Fig. 5) modified from (1).

Knockout mouse experiments indicate that different transcription factors are essential for different stages of haematopoiesis (89). Some of these are indicated in Table 4. Of these a few will be highlighted.

Fig. 5 Growth factors and Transcription factors important in Haematopoiesis. On the right of the pathways, (marked in orange), are growth factors and cytokines important in haematopoietic differentiation. These have been identified both through knock out mouse studies and also by their capability to enhance *in vitro* differentiation down a particular pathway. CFU and BFU stand for Colony and Blast-Forming Unit, respectively. eo- and mega- indicate eosinophil and megakaryocyte/blasts respectively.
Transcription factors which are important at different stages of differentiation/maturation are shown to the left of the pathways, in green. Many of the factors important at the early stages of differentiation (such as Myb and AML-1) are also important for later stage cells, but are marked here next to the earliest stage cells lost by deletion of the transcription factor in knockout studies.


Pluri-potent stem cell
GATA-2
tal-1/SCL
AML-1/CBFβ
Myb
Rbtn-2
Lymphoid stem cell
Ikaros
PU.1
Pre-B
Pre-T
PU.1
IL-7
IL-4
IL-6
Numerous
factors
B
T
IL-3
IL-5
GM-CSF
Myeloid stem cell(CFU-GEMM)
IL-3, IL-1, IL-6, GM-CSF
CFU-GM
BFU-E
mega
CFU-eo
CFU-G
CFU-M
BFU-E
CFU-mega
IL-3/GM-CSF
+
IL-5
PU.1(low)
C/EBP α,ε
eo blast
IL-3/GM-CSF
+
G-SCF
PU.1
C/EBP α,ε
myelo
blast
IL-3/GM-CSF
+
M-SCF
PU.1
C/EBP
mono
blast
C/EBPβ
monocyte
M-CSF
GM-CSF
IL-1+ TNF
GATA-1
EKLF
IL-3/GM-CSF
+
Epo
CFU-E
erythro
blast
P45
GATA-1
NF-E2
IL-3/GM-CSF
+
IL-6/Thrombo
mega
blast

Transcription factor	Phenotype
SCL/tal-1	Lack of yolk sac and definitive haematopoiesis
LMO2/rbtn2	Lack of yolk sac and definitive haematopoiesis
GATA-2	Inhibition of definitive haematopoiesis. Reduced expansion of yolk sac progenitor cells
AML-1	Inhibition of definitive haematopoiesis. Reduced expansion of yolk sac progenitor cells
CBFβ	Reduced expansion of both yolk sac and definitive haematopoiesis progenitor cells
c-Myb	No effect on yolk sac haematopoiesis. 10-fold reduction in all definative lineages except for megakaryocytes which are unaffected
Ikaros	Complete lack of lymphoid lineages
GATA-1	Loss of embryonic and adult erythropoiesis and also block in megakaryocytic maturation
PU.1	Impaired myeloid (granulocyte/monocyte but not erythroid or megakaryocyte) and defective B lymphocyte developmentboth in the yolk sac and in definitive haematopoiesis
Ets-1	Loss of Natural Killer cell lymphocyte lineage
C/EBPα	Defective neutrophil and eosinophil maturation
C/EBPε	Defective neutrophil and eosinophil maturation
C/EBPβ	Impaired macrophage dependent bactericidal effects
NF-E2	Late block in megakaryocytic maturation with thrombocytopenia
HoxA9	Reduced peripheral myeloid and lymphoid compartments

Table 4. Effects of transcription factor disruption by homologous recombination on haematopietic development in the mouse.

Knocking out the tal-1/SCL basic helix-loop-helix factor resulted in a complete lack of haematopoietic cells both in the yolk sac and in the foetal liver. This suggests an important function in the earliest haematopoietic stem cells (173;174). When the gene expressing transcription factor GATA-2 was disrupted, this too was lethal to embryos but whilst a marked lack of haematopoietic cells was apparent, differentiation along the different lineages seemed unaffected suggesting a defect in proliferation of these early progenitors (122). Knockouts of both *aml-1* and *c-myb* genes result in a marked reduction in definitive haematopoiesis, whilst primitive (yolk sac) haematopoiesis remains unaffected (175;176).

Disruption of the GATA-1-expressing gene results in failure of formation of adult red blood cells whilst all other haematopoietic lineages develop normally (15). *In vitro* studies of GATA-1-negative embryonic stem cells show that the block occurs at the pro-erythroblast stage of maturation and results in apoptosis (177). Thus GATA-1 seems to effect primarily a single lineage. Disruptions of other transcription factor genes (e.g. PU.1) correlates with other selective defects in haematopoietic differentiation though more than one lineage may be involved (178).

Whilst expression of a transcription factor may be important in the development of one lineage, down-regulation of the same transcription factor appears to be essential for the development of another as can be seen with GATA-1 in erythroid and myeloid lineages respectively (179).

Like most other tissues, homeobox genes play a large part in the development of haematopoietic lineages. Several genes of the HoxA and HoxB clusters for example HoxA 10, are expressed in haematopoietic cells in a stage specific fashion (180). Primitive pluri-potent progenitors express the more 3' genes of the locus whilst committed progenitors express the more 5' genes.

Together with all these and other lineage-restricted factors, numerous general transcription factors contribute to haematopoietic gene expression including c-myc and c-Jun, Sp1 and NFκB, - reviewed in (181).

3.2 Transcription factors involved in myeloid gene regulation

In myeloid cells, a number of transcription factors are important in the expression of many myeloid genes. These include AML1, the C/EBPs and the Ets factor PU.1(181). These factors together with the predominantly haematopoietic transcription factor Myb and GABP, (another Ets factor that is involved in expression of certain myeloid genes) are discussed further in the next sections since they are the ones I found to be involved in *defensin* gene expression. Other transcription factors also involved in myeloid cell determination include the myeloid specific zinc finger MZF-1 (182) and the retinoic acid receptor protein RARα (183). STATs (signal transducers and activators of transcription) may also play a role in myeloid transcription (184).

Myeloid-expressed genes differ from other tissue specific genes in certain ways. Most myeloid specific genes require only a short upstream promoter sequence in order to be expressed in a tissue-specific manner in transient transfection studies (185). Within this short sequence are the binding sites for a number of the factors listed above. Many such promoters with the exception of those of neutrophil primary granule protein genes lack a consensus TATA box but may have a functional PU.1 binding site at the equivalent position (185).

A single master regulator gene, which causes a switch to a myeloid program once introduced into a heterologous cell, has not been identified. However a

combination of two factors (namely NF-M, a chicken version of C/EBP and v-Myb) has been shown to up-regulate the expression of myeloid genes within heterologous cells (186). Synergism between different transcription factors is now recognised in fact as a a common mechanism of myeloid gene transactivation.

3.3 The Myb family of factors

Transcription factors with a Myb-related DNA-binding domain are found in species as diverse as man, maize and yeast. The functions of these proteins seem to differ in the diverse species as might be expected due to the considerable variation in the proteins apart from this domain. Within mammals, the Myb family of proteins include c-Myb, A-Myb and B-Myb(187). The earliest family member to be discovered, was the viral homolog of *c-myb* gene, the avian myeloblastosis virus oncogene *v-myb*(188). Over expression of *c-myb* is not as related to oncogenic transformation as is *c-myc* over expression, indicating that the structural changes in v-Myb, play a considerable role in its oncogenicity (189). The *c-myb* gene is expressed in haematopoietic cells and in various dividing epithelial cells like those in the colonic crypts (190). During murine development, it is also expressed in foetal thyroid and tracheo-bronchiolar epithelium (191). Other family members include A-Myb (found predominantly in lymphocytes and in the testes) and B-Myb, which is, expressed rather ubiquitously (192).

3.3.1 c-Myb in haematopoiesis

The importance of the c-Myb transcription factor in haematopoiesis was established by a knockout experiment (175). Not much abnormality was apparent in other *c-Myb*-expressing tissues such as hair follicles and intestinal epithelium (which suggests that in these environments B-Myb or some other factor could substitute at least partially for c-Myb). However the gene disruption was pre-natally lethal due to a severe anaemia. Analysis of the affected mice showed normal yolk sac haematopoiesis but severely compromised definitive haematopoiesis with less than 10-fold of the normal amount of erythrocytes and lineage progenitors. All the various lineages were effected except for the megakaryocytes, which were normal in number and morphology. Studies in *Drosophila* show that D-Myb is important in preventing abnormal endoreduplication (193) so megakaryocytic development which requires endoreduplication may be enhanced by the absence of c-Myb. The granulocytes and monocytes appear normal suggesting that it is their quantity which is effected, not their differentiation.

3.3.2 Myb proteins in proliferation and differentiation

c-Myb is expressed most abundantly in primitive proliferating haematopoietic (and other tissue) progenitors (194) in a cell cycle related manner peaking in early S-phase (195). It is down regulated with differentiation of these cells (196). Studies in foetal tissue show that the down regulation of Myb correlates most clearly with terminal differentiation and not with the cessation of proliferation (191). Over expressing c-Myb in myeloid or erythroid cell lines prevents their differentiation in response to various

inducers suggesting that the natural down-regulation seen *in vivo* is a pre-requisite for differentiation (197;198). As well as preventing differentiation, c-Myb expression suppresses apoptosis probably due to c-Myb inducing bcl-2 gene expression (199).

Antisense oligonucleotides directed against c-Myb reduced the number of colonies formed from bone marrow mononuclear cells (200) and inhibited the proliferation of myeloid cell lines and lymphocytes stimulated with mitogen (201;202). All these studies suggest the importance of c-Myb (at least in haematopoietic tissue) for progenitor expansion (proliferation) before differentiation.

3.3.3 c-Myb as a transcription factor

The c-Myb transcription factor has three functional domains (203). These are an N-terminal DNA-binding domain made of three imperfect repeat sequences, a transactivation domain and a negative regulatory C-terminal domain which has structural similarities to a leucine zipper motif (204). Of the DNA binding domain repeats; R1, R2 and R3, (each of which consists of three α-helices), only the R2 and R3 repeats actually form the DNA-interacting portion of the domain. The three α-helices within each repeat are packed around a hydrophobic core made of 3 strongly conserved tryptophan residues resulting in a homeodomain like structure (205). These helices interact with a DNA site with the consensus sequence $AAC^{G}/_{T}G$ (206).

The transactivation domain is hydrophilic and slightly acidic and can activate transcription both within the Myb protein itself or when attached to a heterologous DNA binding domain (such as GAL-4) through the respective binding sites (207). CBP

(CREB-binding protein) binds to this transactivation domain and seems to act as a co-activator for Myb as it does with other transcription factors (208). As well as enhancing the transactivation activities of both c-Myb and the viral oncogenic form v-Myb, CBP also enhances the co-operative effect of Myb with NF-M (a chicken homolog of C/EBP β/ε) on the *mim-1* promoter possibly by producing a co-operative interaction (bridge) between the two proteins (209).

Whilst Myb-induced transactivation can be minimal, Myb co-operates with different transcription factors including Ets and AML family proteins to produce synergistic transactivation of target promoters (210;211) and with C/EBP can even activate myeloid genes in heterologous cell types (186). Such co-operativity does not always involve direct physical interaction between the synergising factors. Myb and Ets factors also co-operate to overcome the effects of the transcriptional repressor ZEB (a mammalian homolog of the Drosophila δEF1 repressor)in haematopoietic cells (212).

Deletions and mutations to the c-Myb protein, as are those found on the transforming v-Myb may also increase Myb-induced transactivation. Both the extreme N-terminus, which contains a phosphorylation site, and the C-terminal regulatory domains reduce Myb-induced transactivation when present. Upon deletion of either terminus, the Myb protein shows enhanced DNA binding which explains the probable mechanism of such enhanced transcription (204). The regulatory C-terminal leucine zipper domain interacts with p160 and p67 proteins, which may play a role in regulating Myb activity (213). Yet another factor which negatively regulates c-Myb activity in myeloid cells is the c-Maf transcription factor which interacts with Myb's DNA-binding domain to form an inhibitory complex which is present most abundantly in immature myeloid cells but which is reduced in more mature cells (214). Thus a whole network of

interactions, both synergistic and inhibitory, characterise the activity of the Myb transcription factor.

3.3.4 Myb-target genes

Myb binding sites are found in the promoter regions of numerous genes. Genes expressed in haematopoietic cells which are known to be regulated by c-Myb, include: the CD4 gene (215), and the *lck* tyrosine kinase (216) which are expressed in lymphoid cells, mim-*1* (211), *c-fms* (217), and *neutrophil elastase* (218) in myeloid cells, and the CD34 gene (219) in haematopoietic stem cells.

Amongst Myb target genes, the human heat shock protein *hsp70* (220) and *bcl-2* (199) promoters are peculiar in that they are up-regulated by Myb in a DNA-binding independent fashion. In the case of *hsp70* this up-regulation depends on the presence of an intact TATA box but this is lacking in bcl-2 suggesting a somewhat different mechanism. Whilst the exact mechanism of transactivation by c-Myb remains obscure, these different mechanisms involved and the activation of different genes by Myb in co-operation with various other factors suggest a complex functional picture.

3.4 The C/EBP family of transcription factors

C/EBP (CAAT/Enhancer Binding Protein) factors were so named upon cloning of the initial family member due to their ability to bind to both the CAAT box in promoters and to a number of viral enhancer sequences. Several family members have since been identified with different transactivating potentials and different expression

patterns. These include the transactivating forms C/EBP α,β,δ and ε as well as the inactive and/or inhibitory forms C/EBPγ and CHOP-10 - reviewed in (221).

3.4.1 Functional domains of C/EBP factors

C/EBPs are transcription factors of the basic zipper family and dimerise by means of their leucine zipper domains. C/EBP dimers bind to non-symmetrical sequences in most cases. In fact though an optimal artificial palindromic binding sequence (GATTGCGCAATC) has been designed (222), natural binding sites are very diverse and usually show significant homology only to half the palindrome. Deletion studies have shown that whilst both the C-terminal leucine zipper and the adjacent basic region are required for good DNA binding, it is the latter which is mainly required for DNA interaction and nuclear localisation whilst the leucine zipper is essential for dimerisation (223). The N-terminal region consists of a transactivator domain (which may be subdivided into separate protein domains) and an adjacent (or intervening in the case of C/EBPα) repression domain or domains (224;225).

The different family members resemble each other in this general structure. The greatest homology between the different family members is within the basic zipper domain(153). This homology allows different family members to interact via their leucine zippers. The amino acids flanking the hydrophobic leucine zipper interface have been proposed to result in preferential matching of certain partners in dimers. Extensive studies of the rat versions of different C/EBPs, showed that whilst all transactivatory family members could homo- and hetero- dimerise with similar affinities, C/EBPε

(crpl) could not heterodimerise well with C/EBPα or β(crp2) but could adequately dimerise with itself or with C/EBPδ (crp3) (153).

One exception to this general co-operative cross-dimerisation is the protein CHOP-10 which has a different basic region and includes helix breaking amino acid residues which prevent it from binding well to DNA. It is known to act as a dominant negative inhibitor of the other C/EBPs probably by interacting with and preventing them from binding their cognate DNA sites(154). Other "inhibitory" C/EBPs include C/EBPγ (Ig/EBP) (159) and N-terminal truncated form of C/EBPα (30kDα as opposed to 42kDa) and C/EBPβ (LIP as opposed to the full-length LAP)(226). All these lack the activation domain and whilst not inhibitory themselves, probably inhibit the active forms by heterodimerising with them or simply by competing for the same DNA binding sites.

Deleting the repression domains of the different C/EBP family members results in up-regulation of the different proteins to different extents. C/EBPα and the somewhat similar C/EBPε (224;225) are up-regulated slightly whilst C/EBPβ(NF-M) is strongly up regulated or de-repressed (227). Once de-repressed but not beforehand, NF-M (the chicken homolog of C/EBPβ) can, in isolation, cause the expression of its myeloid target genes when expressed in heterologous cells as can also be brought about by co-expression with Myb (186). This de-repression can be brought about by phosphorylation of target sites within the repression domain, thus suggesting it to be a physiological process regulating C/EBP function. The similarity of gene activation in heterologous cells by de-repressed NF-M or by NF-M with Myb led the authors of this study to propose that Myb may act in a similar manner by enhancing de-repression.

3.4.2 C/EBPs as transactivators

Many of the C/EBP family members are strong transactivators. Protein elements conserved within the activation domain of these activating family members have been shown to bind to both TBP and TFIIB, both of which are important elements of the RNA polymerase apparatus (228).

The activity of the C/EBP transcription factors can be regulated both by pre- and post-translationally(143) and also by numerous interactions with other transcription factors - reviewed in (221). This results in a very complex system allowing for fine regulatory control in diverse tissues and during different stages of differentiation. As mentioned earlier, using alternate initiation codons, the C/EBPα and β mRNAs can be translated into 'smaller than full length' proteins, C/EBPα 30kDa and LIP respectively (226). Differences in the relative amounts of the two forms produced help regulate the activity by substituting active with inactive forms(229).

Phosphorylation is responsible for a number of post-translational modifications each having different effects on the C/EBPs. Phosphorylation of the C/EBPα basic region (serine 299) attenuates DNA-binding therefore reducing its activity (230). On the other hand, phosphorylation of a threonine residue within an inhibitory domain of chicken C/EBPβ(NF-M), results in transactivation (de-repression) (227). Phosphorylation of a serine (position 276) of the C/EBPβ leucine zipper also results in enhanced transactivation by protein kinase C though the exact mechanism is unclear (231). Such phosphorylation seems also to be important in the functional recruitment of C/EBPβ and possibly also C/EBPδ to the nucleus during differentiation (232). Varying endogenous kinase activity has been hypothesised to be the reason why some C/EBP

members show different activities in different cells. Rat C/EBPs α and β is 10-fold more effective in stimulating the albumin promoter in HepG2 hepatocarcinoma cells than in HeLa cells. (153;233). Of course, these differences may also be due to different factors interacting functionally and/or physically with these C/EBPs in different cell types. A list of some transcription factors interacting with C/EBPs and the genes transactivated by these combinations in shown in Table 5. These include the well-known co-operation of C/EBPs α, β and δ with v-Myb (234) resulting in the expression of myeloid genes *mim-1* and *lysozyme* in heterologous (erythroid or fibroblast) cell lines. The neutrophil elastase gene promoter is also co-operatively transactivated in by Myb together with the activatory C/EBP family members though less with C/EBPβ than the rest (218). It was originally speculated that Myb might somehow de-repress the C/EBP by interacting with its repression domain (227). Recent studies with C/EBPε however, show that the extent of c-Myb dependent co-operation with C/EBP (a two-fold enhancement of activity) is similar with full length C/EBPε or with a repression domain-deleted protein (224).

This indicates that Myb co-operation (at least with C/EBPε) is not mediated by de-repression but by some other as yet undefined mechanism. C/EBPβ and v-Myb have been shown to interact through their DNA-binding domains and this interaction is essential for synergistic activation. This interaction is independent of other proteins (as it occurred between purified proteins) or of DNA (as mutants of v-Myb which could not bind DNA still bound C/EBPβ) (235). Despite their functional synergism and physical interaction, co-operative DNA binding between these proteins has not been demonstrated and synergism was not reduced by separating the distance between binding sites for the two proteins considerably (218).

Transcription factors interacting with C/EBPs	Target genes of the interacting factor combinations
Myb	Mim-1, lysozyme (chicken) neutrophil elastase (human)
ATF proteins	proencephaline
NFκB	Il-6, Il-8
AML-1	M-CSF, defensin
Glucocorticoid receptor	α1-acid glycoprotein
Ets factors Ets1,PU.1, GABP	Eos47(chicken), g-csf, neutrophil elastase

Table 5. Transcription factors interacting with C/EBP factors and some of the target genes co-operatively transactivated by such interacting combinations

Apart from the direct interaction between these factors, they may also interact through their individual interactions with the CBP and p300 proteins (236). Whilst Myb interacts with the CREB-binding site of p300-CBP, and recruits it to DNA, C/EBPβ interacts with the E1A-binding site of p300 (236), possibly resulting in a molecular bridge formed via CBP-p300 between Myb and C/EBP transcription factors. The histone deacetylase activity of CBP, once it has been recruited to DNA may enhance binding of C/EBP by reducing the avidity of nucleosome binding to DNA. C/EBPβ apparently requires loss of nucleosome binding before it can itself bind DNA properly, thus such a mechanism may help explain how Myb co-operates with C/EBPβ in up-regulating target genes in heterologous cells (237).

C/EBPs also interact via their basic zipper regions with the DNA-binding domains of two other transcription factors important in myeloid gene expression. These are the AML-1 protein and the Ets proteins Ets-1 and Fli-1 with which C/EBPα exhibits co-operative DNA binding as well as strong synergistic activation of diverse target genes (238;239). Different C/EBP family members also interact with the myeloid Ets family member PU.1 (240) but this binding is weaker than that with other Ets factors as is the synergism (239).

C/EBPs also interact functionally with a number of other more ubiquitous transcription factors including the retinoblastoma protein Rb (241), c-Jun (242), Sp1 (243), NFκB (244) and NF-Y (245). In this latter case C/EBP actually impedes NF-Y binding but NF-Y enhances the formation of a stable pre-initiation complex around C/EBP therefore stabilising its interaction with DNA. NF-Y has recently been shown to enhance gene transcription by recruiting a CBP-related histone acetyl transferase (246). This suggests that Myb may co-operate in a similar manner with C/EBP, by recruiting

the histone acetyltransferase CBP, and stabilising the pre-initiation complexes around this transactivator.

3.4.3 C/EBPs – expression patterns in different tissues

C/EBP transcription factors are most strongly expressed in liver, adipose tissue, and myeloid cells. The expression pattern and apparent function of the different C/EBPs differs according to the particular tissue. In liver cells, whilst all C/EBPs are up regulated during ontogeny, the ratio of C/EBPα isoforms remains the same whilst the ratio of LAP to LIP (C/EBPβ) proteins increases (226;229). C/EBPα and β are both expressed in terminally differentiated hepatocytes and seem to play a role in constitutive expression of liver genes (226;247). It is the C/EBPβ and δ isoforms, which however play a role in inducible gene expression (248).

In adipose tissue cells, C/EBP β and δ proteins are expressed prior to C/EBPα during adipogenesis (249). In proliferating adipoblasts and pre-adipocytes, C/EBPα is undetectable but it accumulates to considerable amounts in differentiated cells. C/EBPα has been shown to cause growth arrest if expressed prematurely and to initiate adipose tissue-specific gene expression (250;251). Mice with disrupted C/EBPα or both C/EBPβ and δ genes both have defects in adipogenesis and fat metabolism in general (166;252).

3.4.4 C/EBPs in haematopoiesis

Of the haematopoietic lineages, myeloid cells (granulocytes and macrophages) (253), eosinophils (254) and lymphoid cells (B cells) (255) are known to express C/EBPs. Knocking out several of these C/EBPs in mice cause defects in the differentiation and maturation of such lineages though not in actual lineage commitment and initial development of the cells (256-258). Whether the severity of these phenotypes is reduced by functional redundancy is uncertain though certainly possible since different C/EBP family members (α,β,δ,ε) can all trans-activate the promoters of various myeloid genes and co-operate with Myb.

C/EBP α and ε knockout mice show deficits in the differentiation of myeloblasts into granulocytes and in the maturation of eosinophils, whilst C/EBPβ knockouts show defects in macrophage activation. A recent study has also shown that C/EBPε is also very important in phagocytic killing by neutrophils(259). Cells derived from a C/EBPε knockout mouse have deficiencies in the uptake of opsonised bacteria as well as a marked deficiency of secondary granule proteins (260).

Commitment to the myeloid or eosinophil lineages in Myb-Ets transformed multi-potent haematopoietic progenitors depend on the type of C/EBP factor expressed in them. Whilst C/EBPα expression only results in eosinophil cells, C/EBPβ also results in some myeloid cells (254). This difference also probably depends on the interaction with other factors like Ets factors and GATA factors. Ets has been shown to co-operate with C/EBPα (more strongly than with C/EBPβ) to transactivate eosinophil gene promoters(239) so its presence in these cells might direct differentiation away from the myeloid lineage.

C/EBPα is expressed in early myeloid cells and increases transiently during granulopoiesis until the promyelocyte stage after which it is down regulated in terminally differentiated cells (253)(the opposite expression pattern to that seen in adipose tissue). C/EBPε also increases transiently during induced maturation of myeloid cell lines. Whilst C/EBP δ is present in early myeloid cells it is further expressed during G-CSF-induced granulocytic differentiation of multi-potent progenitors (232). C/EBPβ expression is low in dividing cells but increases later on during myeloid differentiation (253).

Changes in nuclear localisation may also regulate C/EBP function in myeloid cells with G-CSF induced maturation of progenitors resulting in functional recruitment of C/EBPβ and δ to the nucleus. C/EBPα on the other hand is present intra-nuclearly throughout its expression in myeloid cells (232).

Numerous genes important for myeloid differentiation, like different cytokines and their receptors, are known to be regulated by C/EBPs and the loss of function of some such genes is critical to the phenotypes seen in knockout mice. Complementation of these phenotypes by replacing such genes can allow some recovery of normal differentiation (261). Some such genes regulated by C/EBPs include G-CSF, M-CSF(185), IL-6(262) and MIP-1α (263).

3.5 The Ets Family of Transcription factors

Ets (E-Twenty-Six) factors form a large family, the first of which to be described being the v-Ets oncogene of the chicken retrovirus E26 (264). Membership in the Ets

family depends on the presence of the ETS domain, an approximately 85 amino acid long motif that is necessary for binding the purine rich core of the DNA target sites of these factors. This family now comprises a large number of transcription factors, homologs of which are found in species as diverse as humans and *Drosophila*. These include Ets-1, Ets-2, Erg, Fli-1, Elk-1, GABPα, Elf-1, Tel and PU.1. - reviewed in (265).

3.5.1 Ets factors, Development and Cancer

Ets genes are expressed in numerous different tissues. Some Ets factors like GABPα and Ets-2 are very widely expressed whilst others can be more selectively expressed such as Elk-1 or ER71 which are predominantly expressed in testis and brain (266). Ets-2 has been shown to be important in meiotic development in Xenopus (267) whilst Ets-1 has been shown to be predominantly expressed in developing mesenchymal cells particularly in angiogenesis (268).

As well as the E26 retrovirus which transforms myeloid and erythroid cells, Ets factors are involved in oncogenesis in a number of different systems. Friend murine erythroleukaemia caused by retroviral infection was found to over express either the *pu.1* or *fli-1* genes in close to 100% of cases, secondary to viral integration just upstream of either gene (70).

Ets factors are involved in chromosomal translocations in some human cancers. In two cases, these translocations involve the ETS domain of the Ets factor being fused downstream of part of an RNA-binding protein. Such a translocation results in a tls/fus-erg fusion mRNA(269) in some myeloid leukaemias and an EWS-Fli-1 protein

(and rarely also an EWS-ERG fusion) in Ewing's Sarcoma - reviewed in Crepieux et al (266). These translocations may alter the activity and/or binding specificity of the native Ets proteins but also enhance the expression of the Ets DNA-binding domains as part of the fusion since these are translocated downstream of a strong promoter. In Ewing's sarcoma, a functional ETS domain is a specific requirement for the oncogenicity of the fusion protein (270).

3.5.2 The ETS DNA-binding domain

Ets proteins bind DNA as monomers, to a site whose consensus sequence is $(^{G}/_{C})(^{A}/_{C})$GGA$(^{A}/_{T})$GT (271). The ETS domain of the Ets proteins is strongly conserved between different family members and shows two areas of particular homology. A C-terminal basic region and a region containing three conserved tryptophan repeats somewhat reminiscent of those seen in the Myb DNA-binding domain (272). Nuclear magnetic resonance studies show the domain formed by three α-helices arranged into a helix-turn helix motif lying against a 4-stranded anti-parallel β-sheet scaffold (273). It was thereby classified according to this structure as a winged helix turn helix (wHTH). The conserved tryptophans form part of a hydrophobic core, which includes residues from all helices, and strands of the β-sheet. This hydrophobic core probably helps stabilise the protein secondary structure. Using the human Ets-1 DNA-binding domain to study protein-DNA interactions, major groove recognition was shown to involve the second helix of the HTH motif. Ets binding resulted in enhanced radical-induced DNA cleavage upstream of the core recognition sequence GGAa/t probably as a result of

DNA bending at this site (271). DNA binding of Ets proteins is regulated by inhibitory interactions between the domains N- and C-terminally adjacent to the ETS domain. These two regions which both contain α-helices co-operate with each other in inhibiting DNA-binding. Deletion of either of these regions increases DNA-binding, as does extensive modification of either such as found in the C-terminal of the viral oncoprotein v-Ets (264). Inhibition occurs through increasing the rate of dissociation of the ETS domain from DNA and involves a direct physical interaction between the two inhibitory domains which probably results in an allosteric alteration of the ETS domain, disrupting binding (274).

3.5.3 Gene Regulation by Ets factors

Ets factors have been described as weak modulators of gene expression in search of a partner (266). Various kinds of transactivation domains are found in different Ets factors, including: the acid transactivation domains of Elf-1 (275), the glutamine rich domains of PU.1 (135), and the proline/serine rich domains of Fli-1, Erg-1 (276;277). A number of known co-operative transactivating partners of Ets factors are listed in Table 6 (266). This co-operativity might depend on previous Ets binding as is the case for GABPα with β (278) or of PU.1 with NF-EM5/PIP (279), or it may depend on previous binding of the co-operative partner like the serum response factor (SRF) in the case of SAP-1 (280). The Ets factor may serve to either recruit the co-operative partner to the DNA or to stabilise the binding of the previously bound factors thus enhancing their transactivation potential. Since the many different kinds of Ets factors all bind to a very similar core recognition sequence in DNA, the diversity of co-operative protein interactions may serve to allow tissue- and stage-specific expression of

target genes depending on the expression of different Ets factors and their particular co-operative partners. This in turn depends on the binding sites for these factors on a particular promoter.

Ets factors often co-operate with other transcription factors in a synergistic manner to transactivate target promoters. In the case of the interactions with core binding factors (CBF/AML) (281) or of GABPα with GABPβ (282), DNA binding co-operativity was also seen to occur by increasing the stability of the DNA-bound complex. Synergising factors may stabilise the Ets-DNA interaction by interacting with one or other inhibitory domain.

Other interactions between Ets and other transcription factors appear more directly involved with activation or inhibition. Ets-1 has been shown to interact with the p300/CBP set of transactivators at two different sites (283). This co-activator complex also interacts physically with other factors, which transactivate genes co-operatively with Ets such as Myb (208), C/EBP (236) and AP-1 (284). Some of these, like Myb, do not interact with the same domain of the CBP protein, as does Ets. Thus CBP may form bridging interactions between these different sets of transcription factors allowing functional co-operation between factors not immediately juxtaposed to each other. CBP may itself play a role in (but appears not to be solely sufficient for) Ets-induced transactivation (283).

A recently recognised inhibitory interaction occurs between Ets-1 and the AP-1 family- related factor Maf-B (285). This factor is expressed exclusively in myelocytic cells within the haematopoietic system, binds via its leucine zipper domain to the Ets-1 ETS domain and form an inactive complex which is DNA-binding independent (285). Over expression of this factor in an erythroblast cell line inhibits erythroid gene expression and differentiation.

Ets Protein	Co-activation partner	DNA-binding co-operativity	Target gene
Elf-1	AP-1	Yes (mutual)	GM-CSF
Erg,Fli-1	E12	No	IgH
PU.1	Pip-1	Yes (PU.1)	Igκ
Elk-1	SRF	Yes (SRF)	c-fos
Ets-1	CBF(AML)	Yes (mutual)	TcRβ chain
Ets-1	Sp1	Yes (Sp1)	HTLV-1 LTR
Ets-1	AP-1	No	uPA
Ets-1	GATA-1	No	$GP_{II}B$
Ets-1	Myb	No	CD13/APN
GABPα	GABPβ	Yes (GABPα)	HSV ICP4

Table 6. Physical and Functional co-operative interactions between different Ets factors and other transcription factors of diverse families. The target genes transactivated by these partners are also indicated.

Ets factors are known to be involved in erythroid differentiation as can be seen by the erythroid transforming properties of the E26 virus as opposed to the predominantly myeloid transforming properties of the AMV virus, which also contains the *v-myb* oncogene but lacks *v-ets*. Ets-1 is expressed in both myeloid and erythroid cells, so the myeloid-specific expression of Maf-B may be involved in preventing differentiation towards the erythroid lineage and stabilising the myeloid phenotype/lineage.

3.5.4 Ets factors in myelopoiesis

PU.1 is the classical myeloid Ets factor but other Ets factors are also expressed in myeloid tissues. These include MEF, Fli-1, Elf-1, (181) and a number of relatively ubiquitous Ets proteins like Ets-2 and GABPα. Whilst Ets-2 is capable of transactivating certain myeloid-specific genes, the myeloid differentiation of embryonic stem cells (ES cells) from which this gene is deleted is not adversely effected, (286) suggesting that it is not essential for gene expression in this tissue. In lymphoid cells, co-operation between PU.1 and other Ets factors is essential in transactivating immunoglobulin enhancer elements (287). Fli-1 (288) seems essential in gene expression and differentiation of erythroid cells. Unlike the case in these other haematopoietic lineages, however, the role of these Ets factors other than PU.1 in myeloid differentiation is unclear at present.

3.5.5 The PU.1 transcription factor

The PU.1 (Spi-1) transcription factor, which has been described as a master regulator of myeloid gene expression, was first isolated from a murine erythroleukaemia (289). Induced expression of PU.1 in multi-potent haematopoietic precursors results in commitment of the cells to the myeloid lineage. A shorter-term expression can however result instead in the formation of eosinophils (290). Together with the closely related Spi-B, it forms a unique Ets sub-family with distinct structures and binding specificity. PU.1 is expressed in murine embryonic stem (ES) cells and in haematopoietic progenitors and is up-regulated during early myeloid development whilst it is down regulated during erythroid development (291). Over-expression of PU.1 in erythroid cells can in fact inhibit their differentiation and may cause apoptosis (17). During differentiation of CD34+ progenitor cells, no further up-regulation of PU-1 expression beyond the promyelocyte stage (291) was detected. Other investigators have shown very high levels of PU.1 m-RNA to be found in macrophages and neutrophils (as well as B lymphocytes) suggesting a further increase may occur during final maturation or activation of these cell types (292).

The *pu.1* gene promoter contains important functional sites, which can bind Sp1, GATA proteins and PU.1 itself (181). This combination of regulatory elements makes for efficient positive feedback thus re-affirming PU.1 expression and the consequent myeloid pathway of differentiation in committed cells.

PU.1-knockout animals die either a few days pre-term or post-term. The major haematopoietic defects seen are a lack of monocytes, neutrophils and B-lymphocytes. Anaemia and loss of T-cells are more variable and depend both on the individual strain of mouse, the severity of the PU.1 gene disruption (178;286) and the duration of

survival of the mice with T cells appearing in the longer surviving animals. The role of PU.1 as an essential transcription factor both in myeloid and lymphoid development is however evident.

3.5.6 Functional domains of PU.1

Like other Ets factors, PU.1 was first recognised by its ability to bind a purine (PU) rich core, hence its name (293). This is a function of its ETS domain. However the DNA binding specificity of this protein was quite different from that of other Ets factors and often (though not always) requires an additional GA adjacent to the usual GGA resulting in a consensus binding site of GAGGA$^{A}/_{T}$.

Many early studies had difficulty in defining PU.1 transactivation domains since it is a relatively weak transactivator but a number have since been identified. These include three relatively strong acidic transactivation domains and a weaker glutamine-rich transactivation domain (the strength of such domains was often tested on artificial promoters in non-myeloid cells) (294). More recent studies have shown that the glutamine-rich domain, together with the DNA-binding ETS domain and the PEST (Proline, Glutamic Acid, Serine and Threonine) domain are all essential for myeloid development unlike the acid transactivation domain (135). These elegant studies used macrophage formation from PU-1 -/- ES cells as a test end-point and studied the required domains by re-introducing different PU.1 mutants and noting which could rescue myeloid differentiation. PU.1 can interact with many other transcription factors. The Ets DNA-binding domain allows interaction of PU.1 with C/EBPδ, (240) CEBPs α and β (239), the runt domain proteins like AML-1 (295) and the c-Jun oncoprotein

(296). The PEST domain allows functional interaction, and co-operative DNA-binding with Pip-1 (NF-EM5), which co-activates lymphoid genes together with PU.1. Mutation of the serine (148) within the PEST domain which inhibits co-operation with Pip-1 but does not inhibit myeloid development suggesting that other residues within this domain are important in this regard. Other proteins which interact with PU.1 include the high mobility group protein HMGI/Y (240), the retinoblastoma protein Rb and TFIID (297).

3.5.7 Transcriptional regulation by PU.1

Experiments using PU-1 -/- ES cells have shown that early myeloid genes such as *myeloperoxidase* and the *gm-csf receptor* are still expressed whilst later myeloid genes like *cd11b* and the *m-csf receptor* are not expressed in the absence of this transcription factor. (298). PU.1 therefore appears to be essential for later rather than early myeloid gene expression. The effects of PU.1 on transcription are dependent to a large extent on its numerous interactions with other factors. In lymphoid cells, PU.1 co-operatively transactivates promoters and enhancers together with Pip-1 or in combinations with other factors including AP-1, high mobility group (HMG) proteins, helix-loop-helix (HLH) factors and other Ets factors (279;299;300).

The importance of these protein- protein interactions is such that in some cases, PU.1 can contribute to transactivation despite lacking an activation domain (300). PU.1 can also functionally inhibit certain transcription factors such as the steroid and retinoid receptors though the relevance of this is as yet unknown (301).

A number of myeloid gene promoters are transactivated by PU.1. These include those of early myeloid genes such as *cd18* (302), the *neutrophil elastase* gene (303) and

the *gm-csf receptor* gene as well of as later myeloid genes such as the *m-csf* gene(185), the *cd11b*(304), and the *macrophage scavenger receptor A* (305). The great majority of these promoters are similar in that they have no TATA box, a pu.1 site close to the site of transcriptional initiation and a number of binding sites for other not exclusively myeloid transcription factors like C/EBPs, AML and Sp1 (306).

Since PU.1 can bind TFIID(297), the first component required for formation of the transcriptional pre-initiation complex, it is speculated that PU.1 may act as a DNA-binding anchor for complex enucleation in the absence of the TATA box. Indeed studies on the myeloid *c-fms* gene promoter detected a number of functional Ets-binding sites near the transcriptional start site, one of which bound PU.1. These sites are sufficient for macrophage-specific basal transcription initiation through the co-operation between PU.1 and other Ets family members (307). Similar studies on another pu.1 site-dependent promoter (that of the human *Fc gamma R1b* gene) showed that insertion of a strong TATA box substituted efficiently for basal and induced transcription initiation in the presence of a mutated pu.1 site (308).

PU.1 can also repress certain genes including the *cd11c integrin* gene (309), and the *I-Aβ* gene (310), possibly through competition with other transcription factors for the same binding sites.

3.5.8 The GA-binding protein

Both the human (E4TF1) and the rat (GABP) forms of this transcription factor were originally identified as proteins binding to cis-sequences required for early gene activation in the Adenovirus and Herpes Simplex type 1 virus genomes respectively

(311;312). Just like other Ets factors and as the name implies, this transcription factor binds a sequence with a purine rich core. This factor was also later recognised to be similar to the mitochondrial gene-binding transcription factor nuclear respiratory factor 2 (NRF-2)(313). This factor was found to be made of more than one sub-unit including a DNA-binding Ets sub-unit (GABPα) and a number of interacting transactivatory sub-units (GABPβ, γ). In the mouse these proteins were found to be expressed from unlinked loci on different chromosomes (GABPα to chromosome 16 and the GABPβ genes to chromosome 2) but which were co-ordinately regulated (314).

3.5.9 Sub-units and domains of GABP

GABP functions as a heteromer, GABPα, a DNA-binding sub-unit which is a member of the Ets family and GABPβ, a transactivatory sub-unit which has a number of ankyrin repeats similar to those found in the *Drosophila* Notch protein (282). The interaction interface between the GABPα and β sub-units includes the part of the Ets domain and the C-terminal tail of GABPα, and the N-terminal domain of GABPβ containing four ankyrin-like repeats. This same GABPβ domain interacts weakly with the DNA, 3' to the core GGAA ets site (278). The C-terminal residues of GABPβ form a coiled coil motif which acts as a dimerisation sequence and can substitute for dimerisation domains of other transcription factors such as a leucine zipper domain (314). GABPβ is purified primarily as a homodimer. Interaction of GABPα with GABPβ and of two GABPβ sub-units together results in the formation of a heterotetramer, a process independent of DNA binding (282). Another protein called GABPβ1–2 or more commonly GABPγ, is probably also expressed from the GABPβ

gene by differential splicing. This splice variant lacks the most C-terminal residues of GABPβ, so that whilst still capable of interacting with GABPα, it cannot homodimerise like GABPβ.

The GABPαβ partnership seems to be an exceedingly specific interaction since GABPβ was found not to augment the DNA binding of the highly similar Ets domains from ER71, ER81 or Ets-1 (315). However most of the residues involved in the GABPα−β interface as identified by crystal structure analysis of the heteromer (316) are well conserved between GABPα and Ets-1, leading the authors to speculate of a possible interaction between Ets-1 and a GABPβ family member.

The avidity of DNA binding of the complex has been intensely studied. It was shown that the GABPαβ complex binds DNA 100-fold more strongly than GABPα alone (127). The GABPαγ complex also binds DNA more strongly but to a lesser extent than the GABPαβ complex. This apparent increase in binding is actually due to a reduced dissociation of the complex from DNA (282). Similar studies with human GABP confirmed this and also showed a markedly strong binding (and protection of DNA from enzymatic digestion) where GABP αβ was bound to more than one adjacent ets site (313).

GABPα expressed in COS-1 cells was not specifically localised to the nucleus but its nuclear localisation was strongly enhanced by co-expression of GABPβ or γ (317). Residues 240-330 are required for nuclear localisation of human GABPβ whilst the activation domain is confined to residues 330-353. Transactivation studies with reporter genes *in vivo* in *Drosophila* Schneider cells were used to define the activation domain. They suggested an overlap between the transactivation domain and the homodimerisation domain of GABP β. This correlates well with the relative lack of

transactivation by GABPγ with GABPα, as well as with the similar results seen with *in vitro* transcription studies (318). Other studies using fusion proteins between GABPβ or γ and the DNA binding/homodimerisation domain of GAL4 show no difference in the transactivation properties of GABPβ or γ. This suggests that it is predominantly the capability to homodimerise and thus localise multiple transactivator domains to the target site by improved DNA-binding which enhances transactivation. These same studies localised the transactivation domain to a glutamine/hydrophobic domain between residues 258 and 237 (132). It was in fact recognised by extensive mutagenesis studies that it was the hydrophobic residues more than the glutamine residues, which were in fact shown to be important for transactivation (319).

3.5.10 Transactivation by GABP

GABP α/β has been shown to transactivate a number of promoters in reporter gene studies. Concomitant with its widespread expression, these include widespread genes involved in the respiratory chain such as the *cytochrome c oxidase sub-unit IV* gene (320). A number of haematopoietic gene promoters such as the *neutrophil elastase* gene promoter (321), the *cd18* integrin gene promoter (322) and that of the γ*c chain* sub-unit of various haematopoietic cytokine receptors (323) are also transactivated by GABP. Synergism with more restricted haematopoietic factors may help explain its tissue-specific role. In the above-mentioned cases, GABP co-operates with C/EBPα and Myb, PU.1 and Sp1 respectively. In all cases, both *in vivo* (as in the studies quoted above) and even in *in vitro* studies (324), both GABPα and β sub-units are required

together in order to cause transcriptional activation. GABPα, with GABPγ, is able to weakly stimulate transcription *in vitro* (324). In promoter sequences, GABP-binding sites can be found just at the transcriptional start site or a considerable distance (40-100bp) upstream (325). In the former cases, a combination of GABP α and β sub-units can initiate transcription in the absence of a TATA box (326). Transcriptional initiation is optimal in the presence of two ets binding sites end to end and spaced by a whole number of helical turns of the DNA helix. This correlates with the preference for GABP binding to a dimeric site. Inhibition of such transcription by antibodies against either GABP sub-unit clearly indicated that GABP could not be substituted by any other Ets factor (325). This correlates with the results of *in vitro* experiments showing the specificity of binding of GABPβ to GABPα (315). There was no such inhibition of transcription in a promoter containing a TATA box as well as a dimeric ets site (325).

Studies on the *cd18* promoter where the GABP-binding sites are somewhat upstream of the transcriptional start site show that even on this promoter, the multiple Ets-binding sites are essential for promoter activity (327). However here, they may not be necessarily involved in transcriptional initiation. Despite the predilection for a dimeric binding site for GABP-induced transactivation, cases where GABP causes transactivation through a single site have been recognised as in the case of the *coagulation factor Factor IX gene* promoter (328). Here GABP binding to a single site adjacent to (and almost overlapping with) C/EBPα is essential for transactivation of the promoter. GABP α/β, a ubiquitously expressed factor and the tissue (liver) restricted C/EBPα co-operate to result in maximal transactivation. GABP also co-operates with PU.1 to transactivate the *cd18* promoter despite the fact that they compete for the same

Ets binding sites (322). In both these cases, the mechanism for such co-operative transactivation is undefined.

3.5.11 Regulation of GABP transcriptional activity

Since GABP is expressed rather ubiquitously (315), its capability to differentially regulate target genes requires modulation of its activity. During early post-natal life, up-regulation of GABPβ expression may play a role in transactivation of certain genes (328). Differential expression of the multiple GABPβ variants (132) in different tissues may also play a major, (if not as yet well defined) role in target gene expression. GABP activity may also be regulated by post-translational mechanisms effecting its dimerisation and DNA-binding potential. Both GABPα and β proteins are targets for casein kinase II phosphorylation (321). Raf-1, which enhances HIV LTR-dependent transcription, enhances the phosphorylation of both GABP sub-units, which then bind to the LTR (329). Insulin up-regulates the *prolactin* gene. However, insulin has been shown to phosphorylate GABPα, which can then bind the *prolactin* promoter and transactivate it. (330).

GABP may also be regulated by redox- sensitive mechanisms, which may be important in respect to its transactivating of a number of respiratory chain enzyme promoters. Reduction of different cysteine residues in the GABPα sub-unit resulted in either inhibition of DNA binding or of its dimerisation potential with GABPβ (331).
Even if GABP is inherently fully active, it can be rendered inactive with respect to many promoters by methylation of cytosine residues within its binding site. This has been

recognised with the mouse M-Lysozyme downstream enhancer (332) as well as the rat thyrotropin receptor gene promoter (333).

3.6 The AML family of Transcription factors

The AML (acute myeloid leukaemia) factors, also known as the core binding factor (CBF) or Polyoma enhancer binding protein 2 (PEBP2) transcription factors, form dimers between a DNA binding sub-unit (CBFα) - of which there are many family members, and a CBFβ sub-unit which does not bind DNA directly but enhances CBFα binding (334). As two of the names imply, these factors were first identified as a result of binding different viral enhancers(335;336). The CBFα sub-unit was also later recognised as a protein involved in one of the most common translocations in acute myeloid leukaemia (AML), hence its third name.

3.6.1 AML factors in haematopoiesis

AML-1 is expressed, though not exclusively, in haematopoietic tissues during myeloid differentiation (337;338). Mouse embryos whose *aml-1* locus has been disrupted die *in utero* due to defective definitive haematopoiesis (176). All lineages are effected including megakaryocytes and possibly also vascular endothelial cells to some extent. This result in a phenotype similar to, but somewhat worse than that seen with the *c-myb* knockout mouse. As in the case of the *myb* knockout, primitive

haematopoiesis was not affected. Disruption of the *cbfβ* gene resulted in an almost identical phenotype (339), upholding the functional inter-relatedness of the two sub-units. It is noteworthy that the other AML isoforms could not effectively substitute for the lack of AML-1.

3.6.2 AML factors and Cancer

AML-1 is involved in one of the most frequent chromosomal translocations in acute myeloid leukaemia (AML), t(8;21)(q22;q22) (340). It is also involved in the t(3;21) therapy-related acute myeloid leukaemia/myelodysplasia or chronic myeloid leukaemia during blast transformation (with AML/MDS1, AML1/EAP and AML1/Evi-1 fusion proteins) and in childhood B-cell Acute lymphocytic leukaemia (TEL/AML1) (181). The CBFβ sub-unit is also involved in a chromosomal anomaly inv (16)(p13;q22) associated with the M4 (FAB classification) variety of AML (341).

Studies on two of the fusion proteins involved in these leukaemias show that the abnormal transcription factors modify normal AML-1 function in both positive and negative ways. They act partly as dominant negatives and partly by stimulating transcription of otherwise inactive genes (alone or synergistically with un-mutated AML-1) (181). This combination of effects leads to a block in differentiation (342) or an abnormal myeloid differentiation (343) in different experimental systems. It seems that, in some cases, the different components of the fusion proteins can act separately and possibly co-operate both to block differentiation and also to stimulate proliferation thus resulting in leukaemic transformation (342).

3.6.3 Functional domains of the AML factors

Multiple CBFα sub-unit genes have been detected (AML-1, 2, 3) and though these are differentially expressed in different tissues, the exact function of each homolog is unclear(344). Both the CBFα and β sub-unit genes are differentially spliced resulting in a number of different forms being detected. The AML-1 gene can be expressed as a result of this differential splicing in either the shorter form AML-1 or the longer AML-1A and B (of these two predominantly as AML-1B). It is these longer forms which are transcriptionally active (345). Such active forms are targeted to the nuclear matrix, an interaction independent of DNA binding and which is missing in the shorter inactive splice variant AML . Such matrix targeting depends on a matrix targeting sequence, which is different from a nuclear localisation sequence and is localised to 31 amino acid residues near to the C-terminus of the protein (346). DNA binding by CBFα proteins depends on the *runt* homology domain. This domain, which is present in all different CBFα splice variants targets DNA with a consensus sequence $TG^{T}/cGGT$ (347).

AML proteins are able to interact with a number of different proteins in most cases through their *runt* homolgy domains. They can physically interact with Ets factors including MEF (348), Ets-1 (349) and C/EBP (295). AML-1 can also interact with co-activators like ALY (350) and co-repressors like Groucho (351). The ability to interact with numerous other transactivators and co-factors means that the effect of AML-1 on different promoters is dependent to a large extent on other associated binding sites within the promoter. Different residues within the *runt* homology domain interact with different factors. Myb and C/EBPα both can synergise with AML-1 through the same

residues but PU.1 requires a more C-terminal part of the *runt* domain (295). AML-1 binds DNA co-operatively with C/EBPα but not with Ets or Myb as a result of such interactions. A specific sub-region of the *runt* homology domain has been recognised as particularly important for Ets factor-interaction (348). These different protein-binding sites may allow interactions with different factors simultaneously, thereby enhancing the formation of multiple transcription factor complexes and functional co-operation.

3.6.4 AML-1 as a transcriptional regulator

AML-1/CBF-β can transactivate certain myeloid promoters alone, such as the *m-csf receptor* promoter (238). However it requires co-operation with other transcription factors to strongly transactivate most target promoters (210;349). This synergism may be dependent on co-operative DNA binding by AML-1/CBFβ with the other factors as is the case when it interacts with C/EBPs through their leucine zipper domain or with Ets-1 through amino acids 123-240 within the Ets N-terminus. C/EBP –AML synergism results in a 60 fold greater transactivation of the *m-csf receptor* promoter(238).

In other cases, however, no clear co-operative binding is detected yet synergism still occurs, as in the case of PU.1 (295) or Myb (352). In most cases synergism is dependent on DNA-binding of both AML and its co-operative partners though this does not always require a precise distance relationship between such DNA binding sites (281). AML-1/CBFβ occasionally forms part of large complexes of transcription factors involving a number of co-activators as happens on the TCRα enhancer. In this case, DNA bending by co-activator LEF-1 can stabilise functional interactions between

AML-1 and Ets-1 thus sponsoring their synergism (353). Myeloid gene promoters up-regulated by AML-1 include many of the primary neutrophil granule protein genes such as *myeloperoxidase, neutrophil elastase* (354) and NP-3 *defensin* (53), making it a crucial transcription factor in early myeloid differentiation.

Apart from co-operative interactions, AML-1 may itself be regulated by phosphorylation of serine residues; such phosphorylation is essential for AML-1 induced fibroblast transformation (355). During granulocytic differentiation, however, it may be primarily regulated by transcriptional methods. In 32D cells induced to differentiate by G-CSF as well as during retinoic acid-induced U937 differentiation, AML-1 is up-regulated early in the process (338;354). Impeding AML-1-induced transcriptional activity during such differentiation, as do many of the AML-fusion products of translocations (dominant negative effect) can easily result in a differentiation block, which may progress to leukaemia.

Aims of the project

Defensin was identified as a gene expressed strongly in certain leukaemic cells as opposed to normal blood (356). This enhanced expression was detected due to the accumulation of cells blocked at a certain stage of differentiation (promyelocytes/myelocytes) as a result of the block of differentiation caused by the leukaemia. During normal differentiation, *defensin* is expressed during a brief window only (51). Therefore , by understanding the regulation of *defensin* gene expression, and identifying the transcription factors involved, it may be possible to identify factors responsible for the elevated level of expression in the leukaemic cells.

Abnormal expression and/or function of transcription factors (often as a result of chromosomal translocations) are known to play a role in leukaemogenesis in a number of cases (81;342). Therefore, understanding normal and leukaemic *defensin* expression may, in the longer term provide insights into transcription factor relationships which may play a role in maturation arrest and thus in causing leukaemia.

A myeloid cell line was used here to study *defensin* expression since it provided an easily available and reproducible source of *defensin*-expressing promyelocyte-like cells. Another benefit of this cell line is that it can be reproducibly differentiated *in vitro* (using chemical inducers), thus recapitulating the process of normal neutrophil maturation to some extent. This allows the study of changes in transcription factor binding with differentiation, and the correlation with defensin gene expression.

The NB4 promyelocytic cell line was used in preference to HL60 because the HL60 cell line maintained in our laboratory had lost *defensin* expression during *in vitro* growth. During initial experiments, another benefit became obvious, namely that NB4

cells were much more easily transfected than HL60s. Previous studies in our laboratory with HL60 cells had identified a number of potentially interesting factor binding sites within the *HNP-3* defensin promoter. However they could not be correlated to mRNA accumulation with differentiation since the cells had lost the ability to express *defensin.*

The initial aims of my study are therefore, to use the NB4 cell line to identify transcription factor binding sites on the *HNP-3 defensin* promoter by *in vitro* footprinting. The importance of these sites will be tested functionally by reporter gene studies and an attempt will be made to identify the factors binding to these particular sites. Using the results of these experiments, I will try to understand the interactions of the different transcription factors with the defensin promoter sequences and how such interactions can diversely regulate defensin gene expression during myeloid differentiation.

Materials and Methods

Chapter 4 : Materials

4.1 Cell lines and tissue culture media

4.1.1 Cell lines

The human promyelocytic leukaemia NB4 cell line, which was isolated by Dr M Lanotte (INSERM, Paris) was obtained from Dr. Ian Traynor (King's College Hospital-University of London). The HeLa and HL60 cells were taken from Beatson Institute laboratory stocks.

4.1.2 Media and sterile supplies

Supplier : Beatson Institute Central Services.

Sterile distilled H_2O	Penicillin (7.5mg/ml)
Streptomycin (10mg/ml)	Amphoteracin B (250μg/ml)
Sterile PBS	Sterile glycerol
Sterile glassware and pipettes	

Supplier: Gibco Europe Life Technologies Ltd., Paisley, Scotland.

L-Glutamine (200mM)	Sodium Pyruvate (100mM)
7.5% (w/v) sodium bicarbonate	
.5% (w/v) trypsin	Special liquid medium (SLM)

Supplier : Northumbria Biologicals Ltd., Cramlington, England.

10x RPMI 1640 medium

Supplier: TCS Biologicals Ltd., Buckingham, UK.

Bovine foetal serum

Supplier : A/S Nunc, Roskilde, Denmark

Sterile tissue culture flasks

Nunc cryotubes

Supplier : Becton Dickinson Labware, Plymouth, Devon, England.

Tissue culture dishes

Sterile Roller Bottles

Supplier : Costar Corporation, Cambridge, Massachusetts, USA.

Tissue culture plates

Supplier : Fisher Scientific International, Loughborough, Leicestershire, England.

Dimethyl Sulphoxide (DMSO)

4.2 Bacterial culture

4.2.1 Media and sterile supplies

Supplier : Beatson Institute Central Services.

L-broth prepared as outlined by Sambrook *et al.* (357).

Sterile glassware

Supplier : Difco, Detroit, Michigan, USA.

Bacto-Agar

Bacto-Tryptone

Supplier : Epicentre Technologies, Madison, WI, USA

Transformation and Storage solution (2X TSS)

Supplier : Sigma Chemical Co. Ltd., Poole, Dorset, England

Ampicillin

Tetracycline

Supplier: Bibby- Sterilin Ltd, Stone, Staffordshire, England

Bacteriological (sterile) petri-dishes

4.2.2 Host cell strains

Three strains of ultra-competent *E.coli* cells were used for transformation of plasmids.

1)*E.coli* host strain DH5α was obtained from Gibco Europe Life Technologies Ltd., Paisley, Scotland

2)*E.coli* host strain Nova Blue was supplied by Novagen

3)*E.coli* host strain HB101 was obtained from a laboratory stock held by Marion Lacey

4.3 Plasticware

Supplier: Becton Dickinson Labware, Plymouth, Devon, England.

Falcon Tubes – 50ml and 15ml (sterile packed)

Supplier: Bibby- Sterilin Ltd, Stone, Staffordshire, England

Sterile 30ml Universal containers and 6ml bijou bottles

Supplier : Du Pont Co., Wilmington, Delaware, USA

Sorvall ultracentrifuge tubes

All other plasticware including pipette tips and microfuge tubes was as supplied by the main laboratory stores

4.4 Water

De-ionised water for buffers and general solutions was obtained from a Millipore MilliRO 15 system. Water for protein/enzyme work or recombinant DNA protocols was further purified by reverse osmosis on a Millipore MilliQ system to 18MΩcm.

4.5 Chemicals

Unless otherwise specified, all chemicals (AnalaR grade) used in the making of buffers or generally in other protocols were obtained from either Fisher scientific international, Loughborough, Leicester, England or from BDH Chemicals Ltd., Poole Dorset, England .

Supplier : Sigma Chemical Co. Ltd., Poole, Dorset, England

Bromophenol blue	Bovine serum albumin Fraction V
Xylene Cyanol	Bicinchoninic acid solution
Ethidium Bromide	Dithiothreitol (DTT)
Spermidine	All-trans retinoic acid (ATRA)

N',N',N',N'-tetramethylethylenediamine (TEMED)

3-(N-Morphlino) propanesulfonic acid (MOPS)

Phorbol Myristate Acetate (PMA/TPA)

p-nitrophenylphosphate (Alkaline Phosphatase Reagent)

Supplier : Premier beverages, Adbaston, Staffordshire,England

" Marvel" non-fat dried milk powder

Supplier : James Burrough Ltd., Witham, Essex, England

Ethanol

Supplier : Fluka Chemika-Biochemika AG, Buchs, Switzerland

Formamide

Supplier : BDH Chemicals Ltd., Poole Dorset, England .

Giemsa histological stain (Gurr)

May-Grunwald histological stain (Gurr)

Supplier : Boehringer Mannheim UK Ltd., Lewes, East Sussex, England

Caesium chloride

Supplier : Gibco Europe Life Technologies Ltd., Paisley, Scotland Enzymes

TRIzol reagent for isolation of RNA

Supplier : Rathburn Chemicals Ltd., Walkerburn, Scotland

Water –saturated Phenol

Supplier : Severn Biotech Ltd., Kidderminster, Worcestershire, England.

Design-a-gel 30%(w/v)acrylamide, 0.8% bisacrylamide solution

Design-a-gel 40%(w/v)acrylamide, 2.1% bisacrylamide solution

Supplier : Mr David Godfrey at Hoffman-La Roche UK

1, 25-dihydroxyvitamin D3 was a kind gift

4.6 Nucleotides and polynucleotides

Supplier: Amersham International plc, Little Chalfont, Buckinghamshire, England

[α-^{32}P] dCTP ~3000Ci/mmol

[γ-^{32}P] dATP ~5000Ci/mmol

[α-^{35}S] L- methionine >1000Ci/mmol

Supplier: Bethesda Research Laboratory, Gibco Ltd., Paisley, Scotland

DNA markers (1μg/μl): 1kb ladder and bacteriophage Φx174DNA (Hae III-cut)

0.24-9.5kb RNA markers (1μg/μl)

Supplier: Sigma Chemical Co. Ltd., Poole, Dorset, England

Yeast tRNA

Salmon sperm DNA

Supplier: Pharmacia Ltd., Milton Keynes, Buckinghamshire, England

Poly (dI-dC)

Ultrapure dNTP Set (100mM)

Supplier: Boehringer Mannheim UK, Lewes, East Sussex, England

Random hexanucleotide mix

Unlabelled nucleotides

Oligonucleotides were made using 'in house' oligonucleotide synthesisers with nucleotide substrates supplied by Cruachem (Glasgow)

4.7 Plasmids

Plasmid	Suppliers
pBLCAT6	M. Boshart (via C.Bartholomew-Beatson Institute)
p0GH	R. Selden (via M.McDonald-Beatson Institute)
pMBM1(Myb)	M.Clarke (via M.McDonald Beatson Institute)
hGH cDNA	Paul Robbins (University of Pittsburgh)
7s rRNA	L. Wu (Beatson Institute)
pcDNA vector	J. Wallin (University of California, Berkeley)
pcDNA-PU.1	J. Wallin (University of California, Berkeley)
pcDNA-N-133 (PU.1)	J. Wallin (University of California, Berkeley)
FNE2full(Ets-2)	Craig Hauser (The Burnham Institute, La Jolla, CA)
pCAGGS vector	Hiroshi Handa (Tokyo Institute of Technology, Yokohama, Japan)
E4FTF1-60 (GABPα)	Hiroshi Handa (Tokyo Institute of Technology, Yokohama, Japan)
E4FTF1-53 (GABPβ)	Hiroshi Handa (Tokyo Institute of Technology, Yokohama, Japan)
E4FTF1-47 (GABPγ)	Hiroshi Handa (Tokyo Institute of Technology, Yokohama, Japan)

pGL2 (basic)	C. Bartholomew (Beatson Institute)
CBFα2-expression	Dr.Nancy Speck (Dartmouth Medical School, New Hampshire)
CBFβ-expression	Dr.Nancy Speck (Dartmouth Medical School, New Hampshire)
pHSV (β-*gal*)	J. O'Prey (Beatson Institute)
pcDNA- C/EBPε	A.Chumakov (Cedars-Sinai Medical Center ,LA)
GAPDH-CAT	M.Alexander (Howard Hughes Medical Institute, Boston, MA)
PSV2Apap	T.Kadesch (via K.Ryan , Beatson Institute)
β–actin (β-*gal*)	Dr H Weintraub (Hutchinson Cancer Research Centre, Seattle, WA)
pBluescript	Promega Inc.
β_2-microglobulin	K. Itakura (via K.Ryan, Beatson Institute)
CHOP-10, CHOP-10-lz	D.Ron (NYU Medical Center, NY)
pSCT-E (Ig/EBP)	Andrew J. Henderson (Cloumbia University, NY)
C/EBPα in pMEX	Pete Johnson (National Cancer Institute - FCRDC, MD);
C/EBPβ in pMEX	Pete Johnson (National Cancer Institute - FCRDC, MD);
C/EBPδ in pMEX	Pete Johnson (National Cancer Institute - FCRDC, MD);

4.8 Enzymes and enzyme inhibitors

Supplier: Bethesda Research Laboratories, Gibco Ltd., Paisley, Scotland

All restriction endonucleases with 10x reaction buffers

Proteinase K

T4 DNA ligase (1U/μl) and 5x ligation buffer

Taq DNA polymerase (10U/μl) and 10x PCR reaction buffer

Supplier: Boehringer Mannheim UK, Lewes, East Sussex, England

Calf intestinal alkaline phosphatase (1U/μl)

RNase A

DNase I

Supplier: Northumbria Biologicals Ltd. (NBL), Cramlington, Northumberland, England

Klenow DNA polymerase (1U/μl)

T4 polynucleotide kinase (10U/μl) and 10x kinase buffer

Supplier: Sigma Chemical Co. Ltd., Poole, Dorset, England

Aprotinin	Leupeptin
Benzamidine	Pepstatin A
Bestatin	Phenylmethylsulphonyl fluoride (PMSF)
Sodium butyrate	β-glycerophosphate
Levamisole	Sodium orthovanadate
Lysozyme	Diethylpyrocarbonate (DEPC)

Supplier : Perkin Elmer Cetus (Norwalk, CT)

"AmpliTaq" Recombinant Taq DNA polymerase

4.9 Antibodies

Supplier : Santa Cruz Biotechnology Inc., Santa Cruz, California, USA

C/EBPα Rabbit IgG polyclonal (14AA)

C/EBPβ Rabbit IgG polyclonal (C-19)

CRP1 (rat C/EBPε-C-Terminal) IgG polyclonal (C-22)

Rabbit anti-mouse IgG, (whole molecule) horseradish peroxidase conjugate.

Anti-C/EBPε (N-terminus) antibody was a gift from Dr A Chumakov (Cedars-Sinai, UCLA).

Supplier : Pharmingen, San Diego, California, USA.

Anti-PU.1 monoclonal antibody (G148-74) raised against a GST-PU.1 fusion protein (whole protein).

PU.1 polyclonal anti-serum against PU.1 was a gift of Dr. Glas (University of California San Diego).

Supplier : Upstate Biotechnology Incorporated, Lake Placid, New York, USA

Anti-c-Myb (C-terminal 235 aa) monoclonal antibody

Phospholipase A_2 monoclonal antibody was a gift of Dr. Angeliki Malliri (Beatson Institute).

Supplier: Amersham International plc, Little Chalfont, Buckinghamshire, England

Donkey anti-rabbit IgG, (whole molecule) horseradish peroxidase conjugate.

Purified GABPα and GABPβ proteins synthesised in and purified from *E.coli*, as well as polyclonal rabbit antiserum raised against these two proteins, were kind gifts from Dr Tom Brown (Pfizer, Groton CT).

4.10 Membranes, papers and radiographic film

Supplier: Amersham International plc., Little Chalfont, Buckinghamshire, England

Hybond N nylon membrane (fingerprint grade)

Supplier: Millipore Corporation, Bedford, Massachusetts, USA

Immobilon membranes

Supplier: Whattmann International Ltd., Maidstone, Kent, England

3MM filter paper

Supplier: Eastman Kodak Co., Rochester, New York, USA

(X-OMAT-AR) X-Ray film

Supplier: Fuji Photo Film Co., Tokyo, Japan

(RX) X-Ray film

Supplier: Sartorius AG, Gottingen, Germany

Colloidon dialysis bags

4.11 Molecular Biology Kits and Columns

Supplier: Amersham International plc, Little Chalfont, Buckinghamshire, England

ECL Western Blotting Analysis System

Suppler: Applied Biosystems, Warrington, UK

Dyedeoxy Terminator Cycle Sequencing Prism Kit

Supplier: Qiagen Ltd., Dorking, Surrey, England

QIAquick Gel Extraction Kit

QIAgen EndoFree Plasmid Maxi Kit

Supplier: Nichols Institute Diagnostics., Saffron Walden, Essex, England

Human Growth Hormone RadioIsotopic Assay Kit

Supplier : Pharmacia Ltd., Milton Keynes, Buckinghamshire, England

Sephacryl S-400 HR resin MicroSpin columns

NICK columns

Supplier : Perkin Elmer Cetus (Norwalk, CT)

GeneAmp DNA Amplification Reagent Kit with AmpliTaq.

Supplier: Promega, Madison, Wisconsin, USA

TNT T7 Quick Coupled Transcription/Translation System

Supplier : Sigma Chemical Co. Ltd., Poole, Dorset, England

Naphtol AS-D Chloroacetate and α-Naphthyl Acetate Esterase diagnostics kit

Nitroblue Tetrazoilium Reduction kit

4.12 Equipment

Suppler: Applied Biosystems, Warrington, UK

ABI 373A DNA Sequencer

Supplier: BioRad Laboratories, Richmond, California, USA

BioRad Gene Pulser with capacitance extender

Supplier: Flowgen Instruments, Ltd., Lichfield, Staffordshird, UK

4mm chamber Electroporation cuvettes

Supplier : Beckman

Gamma 5500B γ-counter

Chapter 5 : Methods

5.1 Cell culture

5.1.1 Culture conditions during routine passage of cell lines

All cell lines were incubated at 37°C in humidified air with 5% added CO_2. NB4 cells were grown in RPMI 1640 supplemented with 12% foetal calf serum, 5.0 units of penicillin and 5µg of streptomycin per ml. Cell cultures were maintained at a density of 2-10x10^5 cells/ml and 2 days prior to transfection they were cut back to 4x10^5cells/ml. Cells used during experiments were from passage 4 till 12.

HL60 cells were passaged every 2 days in routine culture with cells being cut back to concentrations of 5x10^5 cells/ml. HeLa cells were passaged twice weekly in routine culture with new cultures being re-seeded with 1x 10^6 cells.HeLa and HL60 cells were grown in Special Liquid Medium supplemented with 10% foetal calf serum and glutamine (final concentration 2mM).

5.1.2 Cryopreservation and recovery of cells

Cells to be stored at a particular passage number were counted. They were then pelleted by centrifugation at 350 x g for 10 minutes at 25°C and re-suspended at a concentration of about 10^7/ml in 90% serum 10% DMSO(for HL60 and NB4 cells) or 90% serum supplemented medium, 10% DMSO for HeLa cells. 1ml of the cell

suspension was aliquotted into 1.5 ml Nunc cryopreservation tubes and frozen slowly overnight in a protective polystyrene box to -80°C. The frozen samples were later submerged in liquid nitrogen storage Vats where they were stored long term.

To thaw cells, samples were placed in a 37°C water bath till thawing, washed with 10 to 20 ml of fresh medium, pelleted by centrifugation at 350 x g for 10 minutes at 25°C, and resuspended in the required volume of new medium.

5.1.3 Mycoplasma testing

Hoechst 33258 staining to exclude Mycoplasma contamination was performed as described by T.R.Chen (358). 2ml of medium from the cell line being tested were incubated with 10^4 NRK cells(an indicator cell line) in 60mm petri-dishes with 4 ml fresh medium for 3-4 days. The medium was then removed, the cells washed repeatedly with PBS, and fixed on the dish. 5mls of Hoechst 3258 stain in PBS (0.05μg/ml) was added and left for 10minutes. The stain was removed, the cells washed twice in distilled water, mounted in McIlvaine's buffer and sealed. They were then viewed with a fluorescence microscope. Any fluorescence seen outside the nucleus is due to extra-nuclear DNA and is indicative of Mycoplasma infection.

5.1.4 Induction of differentiation

NB4 cells were differentiated by addition of inducers to a final concentration of 1μM all-*trans*-retinoic acid (ATRA) for granulocytic differentiation (359), and 0.4μM

of each of 1,25 di-hydroxy cholecalciferol (vitamin D3) and phorbol myristate acetate (PMA) for monocytic differentiation (95).

5.1.5 Cell Staining - May-Grunwald and Giemsa stains

1x 10^6 NB4 cells were harvested by cytocentrifugation at 350 x g for 5 minutes onto a slide. They were fixed by flooding with methanol for 5 minutes at 25°C. The slides were stained with May-Grunwald stain (1 part stain in 2 parts Sorensen's buffer). Sorensen's buffer is 0.066M Na_2HPO_4 and 0.066 M KH_2PO_4 pH6.85. The slides were then transferred to Giemsa stain (5% in Sorensen's buffer) for 10 minutes at 25°C. They were then washed twice in distilled water, mounted, and left to dry for 24 hours.

5.1.6 Nitroblue tetrazolium staining

Granulocytic differentiation of NB4 cells was tested using NBTZ staining by a modification of the "stimulated" test procedure described by the manufacturer with the testing kit provided (see Materials section 4.11). 0.25 ml of NBTZ solution were added to a vial with a plastic pipette. In place of the supplied stimulant solution, 5μl of Phorbol Myristate Acetate (PMA) at a concentration of 1mg/10ml (in alcohol) was added as stimulant. 1x 10^6 NB4 cells resuspended in 2 ml of PBS were added and incubated together with the NBTZ/PMA mixture for 30 minutes at 37°C and then for an additional 10 minutes at 25°C. 0.5 ml of this cell suspension in NBTZ solution was then transferred to a slide by means of cytocentrifugation. The cytospun cellular preparation

on the slide was stained with Wright stain (provided) for 15 seconds, with diluted stain (flood the slide with distilled water) for a further 30 seconds, after which the stain was washed off. The slide was then dried, mounted and viewed by microscopy.

5.1.7 Non-specific Esterase staining

NB4 cells were assessed for monocytic differentiation by non-specific esterase staining using the α-Naphthyl acetate esterase procedure as described by the manufacturer in the supplied test kit. 1ml of Sodium Nitrite solution was added to 1 ml of fast Blue BB Base, mixed and allowed to stand till the colour converts from brown to a deep yellow. 40 ml of pre-warmed (37°C) de-ionised water was added to this solution. Furthermore, 5 ml of TRIZMAL 7.6 buffer concentrate was added as well as 1 ml of α-Naphthyl acetate solution. The solution turns green. Slides prepared with 1x 10^6 NB4 cells from differentiation time points were prepared previously and now fixed in Citrate Acetate Formaldehyde solution at room temperature (23 to 26°C) for 30 seconds with vigorous agitation in the last 5 seconds. The slides were rinsed in running de-ionised water for 1 minute and then incubated in the previously prepared green solution for 30 minutes at 37°C, shielded from light. The slides were then washed for at least 2 minutes in running de-ionised water, counter stained with Hematoxylin solution, washed in tap water, air dried, mounted using an aqueous mounting agent and viewed by microscopy.

5.2 Transient Transfections and Reporter Gene Assays

5.2.1 Plasmids for Transfections

The human growth hormone (hGH) reporter gene construct was made by cloning hGH cDNA into the CAT vector pBLCAT6 (360) in place of the CAT cDNA and improved to reduce vector-dependent promoter effects. Many defensin deletion mutant promoter fragments were obtained from A.C. Philips (Beatson Institute). Others were amplified by PCR using primers with appropriate Hind III and Bam HI restriction endonuclease sites at their ends. Amplified sequences were cloned into restriction sites on the reporter vector. Site-specific mutations were introduced using a method previously described (361). All PCR-derived cloned promoter sequences were verified by sequencing. The GAPDH, β-actin and herpes simplex thymidine kinase promoter sequences were directly cloned into the hGH reporter gene using available restriction sites.

5.2.2 Electroporation

Transient transfections into undifferentiated and differentiated NB4 cells followed exactly the same protocol. For the 2 days prior to transfection they were cut back to $4x10^5$cells/ml. This increases the percentage of cycling cells prior to transfection. Differentiated NB4 cells were treated with inducer 20 hours prior to transfection. $7x10^6$ cells were harvested by centrifugation at 350 x g for 10 minutes at 25ºC and their conditioned RPMI 1640 medium reserved. The cells were then suspended in 200μl of special liquid medium (SLM) supplemented with foetal bovine

serum and glutamine. Plasmid DNA for transfection, (5 μg of reporter vector, 5 μg of internal control, any expression vectors and made up to a total of 30μg with pBluescript DNA) was prepared in a final volume of 50μl sterile 1xTE (1x TE is 10mM Tris.HCl , 1mM EDTA - pH8.0). This was mixed with the concentrated cell suspension in a 4mm gap electroporation cuvette (Flowgen) and the cells were electroporated at 960μF and 200V using a Bio-Rad gene pulsar. 1ml of fresh SLM medium was immediately added, and the contents of the cuvette transferred to a 60mm diameter dish with 4ml of conditioned RPMI medium. After recuperative incubation at 37° C in a humid atmosphere with 5% CO_2 for 20 hours, two 100μl aliquots of medium were removed for analysis.

HL60 cells are electroporated in a similar manner as are NB4 cells. Differences include that cells are grown in SLM not RPMI and are cut back to 5 x 10^5 on the 2 days prior to transfection. Electroporation is performed using a total of 50, not 30 μg of DNA and at 250 not 200 V.

5.2.3 Calcium Phosphate transfection

HeLa cells were seeded at $1x10^6$ cells per 60mm dish and incubated overnight prior to transfection. The DNA to be transfected (5μg of reporter construct, plus expression constructs, and pBlueScript carrier DNA to a total of 10μg) was diluted in filter-sterilised water. $CaCl_2$ was added to a final concentration of 0.22M in a final volume of 1ml. This solution was added drop-wise to an equal volume of 2 x HEPES-buffered saline (280mM NaCl, 50mM HEPES, pH7.1, 1.5mM Na_2HPO_4) and incubated for 30 minutes at 25°C. Medium was aspirated off the cells and 2ml of transfection

mixture together with 4ml of fresh SLM medium added. After overnight incubation for recovery, medium was removed, fresh medium added and, after a further 24 hours, a sample of medium was removed for reporter gene assay.

Since alkaline phosphatase (SV40 early promoter) reporter activity was drastically affected in HeLa cells by co-expression of some of the transcription factors, no internal control was included in HeLa transfection experiments since including the alkaline phosphatase activity would have invalidated the results. Therefore, multiple transfections (at least 4 for each experiment) were carried out.

5.2.4 Assay for Placental Alkaline Phosphatase

Alkaline phosphatase activity was determined as has been previously described (362) using 100µl of a 1:10 dilution of the medium for NB4 cells. With HL60 cells 100µl of medium are used directly since the activity produced following transfection is much less. Any cells are pelleted by centrifugation. The sample was heated at 65°C for 2 hours in order to degrade any endogenous (heat labile) alkaline phosphatase activity (secreted placental alkaline phosphatase activity is heat labile). It was then cooled on ice. 1 ml of substrate (5mM p-nitrophenylphosphate in DEA buffer) was added to the sample and the reaction transferred to 37°C. Absorbency at 405nm was measured when an orange/yellow colour begins to develop after twenty minutes. DEA buffer is 1M diethanolamine, 0.28M NaCl and 0.5 M MgCl, pH 9.85 and stored at 4°C.

5.2.5 RadioImmunoassay for human Growth Hormone

Growth hormone was measured in one aliquot by radioimmunassay (Nichols Institute Diagnostics, CA) according to the manufacturers' instructions. 100µl of medium (from which the cells had been removed by centrifugation) was added to 100µl of anti-human growth hormone antibody mixture in a clean round bottomed polystyrene tube. The antibody mix included an ^{125}I-radiolabelled antibody against one growth hormone epitope and another biotin-linked antibody against another epitope. The solutions were carefully mixed by gentle vortexing. An avidin coated bead was added and the tubes incubated with gentle circular shaking for 4 hours at 25°C. Upon incubation, this results in the formation of a BEAD-avidin-biotin-Ab-hGH-Ab-radioisotope sandwich. After this incubation, the supernatant is aspirated, the bead washed thoroughly twice with the provided wash buffer, and the bead then transferred to a suitable vial and gamma irradiation counted in the Gamma 5500B Beckman counter.

5.3 Molecular Biology

5.3.1 Microbiological techniques

5.3.1.1 Generation of competent cells

E.coli (of whatever strain) grown overnight, were diluted 1:50 with LB broth. They were incubated at 37°C with shaking (at 200 rpm) until the cells reached early log phase (OD_{600} = 0.25-0.4). 2x Transformation & Storage Solution (Epicentre Technologies) was diluted in the required amount 1:1 with sterile distilled water and the 1x solution was chilled on ice. (100μl of this solution is required for 1 ml of cells). 1 ml aliquots of the log phase growing cells were pelleted in sterile microfuge tubes by centrifugation for 1-2 minutes at 4°C. The supernatant was removed by a sterile pipette tip and 100μl of cold 1x TSS solution was added and the pellet gently resuspended by pipetting. The cells were either transformed (as described below) or frozen immediately in a dry ice/alcohol bath and stored frozen at -70°C to maintain their competency.

5.3.1.2 Transformation of bacterial cells

Frozen aliquots of competent cells were thawed slowly on ice. 100pg –10ng of DNA required to be transformed into the cells was added to each tube, which was then flicked to mix the cells and incubated for 10 minutes on ice. The tubes were then

transferred to 25° for a further 10 minutes. Once again the tubes were transferred to ice and incubated for a further 10 minutes. 1 ml of LB broth was added and the cells were incubated at 37°C for at least 1 hour with shaking after which the transformed cells could be selected by plating on a selective medium to identify incorporated resistance genes in the transformed plasmids.

5.3.1.3 Preservation of transformed and host strains

The DH5α, Nova Blue and HB101 *E.coli* cells transformed with useful plasmids were stored as glycerol stocks for future retrieval. Stationary cultures in liquid medium were mixed with glycerol resulting in a final concentration of this antifreeze agent of 30% (v/v) /L-broth solution (see Section 4.2.1), cooled on ice and then frozen at -20°C.

5.3.2 DNA isolation from bacteria

5.3.2.1 Minipreparation of plasmid DNA

One step 'miniprep' method for the isolation of plasmid DNA was carred out according to Chowdhury (363). 1.5ml of overnight bacterial culture in L-Broth (see section 4.2.1) was harvested in a microfuge tube and spun at 14,000rpm in an Eppendorf microfuge for 30sec. 1ml of supernatant was discarded, 0.5ml of PCI (phenol:chloroform:isoamylalcohol = 25:24:1) added and the mix vortexed and then transferred to a Eppendorf mixer for 5-10min. Samples were spun at 20°C for 5min and the upper aqueous layer transferred to fresh microfuge tubes (phenol chloroform

extraction) containing an equal volume of isopropanol. After mixing thoroughly by vortexing, plasmid DNA and RNA was pelleted by centrifugation as above for 10min. The pellets were washed twice with ice-cold 75% (v/v) ethanol, air dried and re-suspended in 20µl of 1xTE (see section 5.2.2) containing 20µg/ml RNaseA.

5.3.2.2 Large scale preparation of DNA - purification by ultracentrifugation

500ml of overnight bacterial culture of *E.coli* in L-broth (see section 4.2.1) were harvested and spun at 4,000rpm for 10min at 4°C using a Sorvall Centrifuge RC3C using a H6000A/HBB6 swing-out rotor. The supernatant was discarded and the bacterial pellets re-suspended in 5ml Resuspension Buffer (50mM Tris.HCl (pH8.0), 10mM EDTA (pH8.0), and 100µg/ml RNase A), mixed well by vortexing, and then 10ml of Lysis Buffer (200mM NaOH and 1% SDS) added, and the tubes were inverted gently for a few times to achieve a homogeneous lysate without shearing the bacterial genomic DNA. Then 7.5ml of Neutralisation Buffer (3M K^+Acetate [pH5.5]) were added, mixed well, and centrifuged at 8000rpm for 5 minutes at 4°C. The supernatant was filtered through 2 layers of gauze, 0.6 volume of isopropanol added, and the contents mixed by vigorous shaking before pelleting the plasmid DNA and bacterial RNA by centrifugation at 8000rpm for 5 minutes at 4°C. DNA of this quality, whilst suitable for transfection into certain cells once RNA had been removed is considerably toxic to myeloid cells during transfection, possibly due to the presence of lipopolysaccheride from the bacterial cell walls, a myeloid cells stimulant. For this reason further purification was required. The pellet was therefore dried in air, after which it was dissolved in 7.4 mls of TE (see section 5.2.2) buffer. 7g of CsCl was added together with 300µl of Ethidium Bromide (10µg/ml). The refractive index was

measured and CsCl or TE was added as required to obtain a final index of between 1.3890 and 1.3915. After accurately balancing the Ti 60 tubes, containing this solution, they were ultracentrifuged at 40,000 rpm for 40 to 60 hours at 20°C. After centrifugation, the double stranded super-coiled DNA band was extracted from the CsCl gradient and cleaned from ethidium bromide by repeated isopropanol extractions using TE-equilibrated isopropanol until the aqueous phase was clear. This DNA solution is dialysed in collodion bags against 1x TE buffer (see section 5.2.2) for 16 hours at 4°C changing the buffer at least 3 times. This allows removal of the casium salts which are toxic to the cells. The content of the collodion bags was then ethanol precipitated with a 0.1 volume of 0.3M sodium acetate and 2.5 volumes of ethanol overnight at –20°C. The precipitate was washed with cold 70% ethanol, air dried, and re-dissolved in TE whereupon it was quantified by spectroscopy as described in section 5.3.4. This method (which can be scaled up to use even larger starting volumes of bacterial super-broth culture) was used when huge amounts of a particular plasmid was required, such as the pBluescript carried plasmid or the alkaline phosphatase internal control plasmid.

5.3.2.3 Large scale preparation of plasmid DNA-purification by QIAgen

This method of preparation was used when large amounts of a plasmid were required but not the huge amounts required as for plasmid used in most transfections. Large scale bacterial growth was allowed to occur as described above, though lesser volumes of bacterial culture were required with only 100ml of bacteria transformed with a high copy plasmid being used with each Maxi prep Qiagen column. The bacteria were

pelleted after overnight growth by centrifugation at 6000rpm in a Sorvall GS3 rotor. The pellet was then resuspended in 10 ml of buffer P1 (to which RNAse A had been added to a concentration of 100μg/ml). This was continued till no particular clumps of bacterial cells could be seen. 10ml of buffer P2 were added, the solutions mixed by inverting the centrifuge bottle for 5 times, and incubation allowed for 5 minutes at 20°C. This results is cellular lysis with the suspension turning viscous. A QIAfilter Cartridge was prepared by screwing a cap into the outlet nozzle. 10ml of chilled (ice) Buffer P3 was added to the lysate and mixed immediately by inverting the bottle 5 times. The lysate following such mixing was immediately poured into the QIAfilter cartridge and allowed to stand for 10 minutes at 20°C. The cap was removed from the QIAfilter Cartridge, the plunger inserted and the lysate filtered into a clean sterile 50 ml tube (Falcon). 2.5 ml of buffer ER (endotoxin removal) was added to the lysate (approximately 25 ml), the tube inverted for 10 times and incubated on ice for 30 minutes. A QIAGEN-tip 500 was equilibrated by applying 10 ml of buffer QBT and allowing the column to empty by gravity flow. The lysate was applied to the column and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed twice in succession with 30mls of buffer QC. The DNA was then eluted with 15 ml of buffer QN. The DNA was then precipitated by the addition of 10.5 ml (0.7 volumes) isopropanol (25°C), mixing immediately by inverting the tube 4 or 5 times, and immediate centrifugation at 11000 rpm for 30 minutes at 4°C in a Sorvall SS-34 rotor. The supernatant was carefully decanted off, washed with 2.5 ml of endotoxin free 70% ethanol (made using supplied endotoxin free water), and centrifuged at 11000 rpm for 10 minutes at 4°C in a Sorvall SS-34 rotor. The supernatant was again carefully decanted, the pellet air-dried for about 5 to 10 minutes, without allowing overdrying

(since this makes dissolution very difficult). The pellet was dissolved in endotoxin free TE (supplied) and quantified as described (see section 5.3.4).

5.3.3 Recombinant DNA techniques

5.3.3.1 Restriction enzyme digestion

Small quantities of plasmid DNA (usually < 2μg) were digested in the appropriate buffer in a total volume of 20μl using 1-10 units of enzymes per ug of DNA, depending on the enzyme used and the number of sites present. Larger, preparative digests were carried out using proportionately larger volumes. For double digests, suppliers' information was consulted and the most appropriate buffer used. Reaction mixes were incubated for 2-3h at 37°C and then terminated by the addition of 1/5th volume of 6x DNA gel-loading buffer (6x buffer: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% glycerol).

5.3.3.2 Agarose gel electrophoresis

DNA fragments were resolved on agarose gels containing ethidium bromide for subsequent visualisation under UV luminator. In general, 1% (w/v) agarose gels were used, but smaller fragments (100-400) were separated on 2-4% gels. Gel mixes containing the appropriate amount of agarose in 1 x TBE (90mM Tris, 90mM boric acid and 2mM EDTA - pH8.0) were heated in a microwave oven to

dissolve the agarose, and then cooled to 60°C when ethidium bromide was added to 0.5μg/ml and the mix was poured into appropriate gel cast. Gels were allowed to set at room temperature and installed into the electrophoresis tank in 1xTBE buffer. Samples containing the DNA gel-loading buffer (see section 5.3.3.1) were loaded and electrophoresed at around 100V. Molecular weight standards used include the 1kb ladder and Hae III-digested bacteriophage Φx174 fragments. A photograph was taken of the gel with a polaroid camera, using a TF-35M UV luminator (Vilber Laurmat).

5.3.3.3 Purification of DNA fragments from agarose gels

DNA fragments excised from agarose gels were purified with the QIAquick gel extraction kit. The required bands were excised with a clean sharp scalpel. The gel slice was weighed and 3 volumes of buffer QX1 were added to one volume of gel (300μl buffer for 100mg of gel). The mixture was incubated for 10 minutes at 50°C with intermittent flicking to help dissolution of the gel. The melted gel sample was loaded into a Qiaquick spin column which was itself placed into a 2 ml collection tube. For large DNA fragments (more than 5kb) 1 gel volume of TE buffer (see section 5.2.2) was added to the solubilised gel slice and mixed by inverting prior to loading on the QIAquick column. The column/collection tube set-up was centrifuged at maximum speed in an Eppendorf microfuge for 60 seconds. The fluid collected in the collection tube was discarded and the column returned to the same tube. Washing was performed with 0.75 ml of Buffer PE which was added to the column prior to another 60 second centrifugation. When the fragment was required for blunt end ligation, the column was allowed to stand for 5 minutes with the washing buffer PE, prior to centrifugation. Wash buffer was also discarded and the column was again centrifuged in the collection

tube to remove any residual traces of PE buffer. To elute the DNA, the column was placed in a clean 1.5 ml microcentrifuge tube. 50 μl of 10mM Tris-HCl [pH8.5] or double distilled water was added and the set-up centrifuged for 60 seconds. The DNA was now ready for further manipulations.

5.3.3.4 Phosphatase treatment of vectors for ligation

The required vector was digested to completion (long incubation with excess of required restriction endonuclease enzyme) in a total volume of 50 μl. 1μl of calf intestinal alkaline phosphatase (CIP was added and incubation was allowed to proceed for 1 hour at 37°C in the same restriction enzyme buffer. 6μl of 10% SDS and 6μl of 10x STE (100mM Tris pH8, 1M NaCl, 10mM EDTA) were added and the reaction then incubated at 68°C for 15 minutes. The aqueous phase was extracted by phenol chloroform extraction (see section 5.3.2.1) and transferred to a fresh tube. 2μg of transfer RNA was added and the DNA was precipitated with $1/10^{th}$ the volume of 0.3M sodium acetate and 2 volumes of ethanol on dry ice for an hour. The DNA was pelleted by centrifugation in an Eppendorf microfuge at full speed for 10 minutes. The supernatant was aspirated, the pellet washed with 70% ethanol at –20°C and centrifugation repeated. The supernatant was removed, centrifugation repeated and the remaining alcohol removed. The DNA was resuspended in TE (see section 5.2.2) at an approximate concentration of 10ng/μl and the actual concentration was then assessed on an ethidium bromide stained agarose gel using HaeIII ϕ X174 digest for comparison.

5.3.3.5 Ligation reactions

10 to 20 ng of dephosphorylated vector was added together with a 3 fold molar excess of insert and 2µl of 5x ligation buffer and 1 unit of T4 DNA ligase (Gibco BRL). Water was added to a total of 10 µl and incubation was performed overnight at 16°C (for blunt ended ligations) and 4°C (for sticky end ligations). The ligated vector was then transformed into competent cells and selection performed using the appropriate antibiotics.

5.3.4 Quantitation of nucleic acids

Nucleic acid concentrations were determined spectrophotometrically. 3µl of the sample was added to 300µl of dH_2O and absorbance (A) readings taken at 260nm and 280nm in a quartz cuvette using a DU 650 spectrophotometer (Beckman). dH_2O was used as the blank for quantitating DNA samples. The blank for RNA samples was prepared by adding 3µl of RNA dissolving buffer into 300µl dH_2O. An A_{260} value of 1 was taken to be equivalent to 50µg/ml of plasmid or genomic DNA, 40µg/ml of RNA and 20µg/ml of oligonucleotide. The A_{260}/A_{280} ratio was used as a measure of purity: samples giving a ratio less than 1.75 were further purified by Phenol/Chloroform extraction (see section 5.3.2.1), ethanol precipitation and the A_{260}/A_{280} ratio was then re-assessed.

5.3.5 Sequencing of DNA

PCR products were sequenced using cycle sequencing following cloning into the appropriate vector. They were sequenced on both strands using the same primers used in the initial PCR reaction. 3.2 pmols of primer was added to approximately 1μg of plasmid DNA and the total volume made up to 12μl with RQ grade ddH_2O. To this mixture was added 8μl of the Dideoxy Terminator Cycle Sequencing Prism Kit (Applied Biosystems). Reactions were carried out in a Perkin-Elmer 9600 thermal cycler using using 25 cycles 96°C for 15sec, 50°C for 1sec and 60°C for 4min or in a Perkin Elmer Thermal Cycler 480 using 25 cycles 96°C for 30sec, 50°C for 15sec and 60°C for 4min. The resultant DNA products were precipitated (see Section 5.3.2.1) and dissolved in 3-4μl of loading buffer (5x: 5 parts deionised formamide and 1 part 50mM EDTA containing 30mg/ml blue dextran). Running and analysis of sequencing gels was expertly performed by Robert MacFarlane (Beatson Institute, Glasgow, UK) as follows. Samples were run on a 6% polyacrylamide gel (made from a stock with 40% acrylamide and 2.1% bis-acrylamide) in 1xTBE buffer TBE (see section 5.3.3.2) using an Applied Biosystem ABI 373A DNA Sequencer at 30W for 12h. Gels were analysed using 373A software version 1.2.1.

5.3.6 Polymerase chain reaction (PCR)

Amplification of fragment from plasmid DNA was carried out by polymerase chain reactions (PCR) using *Taq* DNA polymerase. The concentration of DNA template

used in PCR reactions was 1ng/μl for plasmid DNA in 1x TE (see section 5.2.2). 2μl were used for amplification. The PCR reaction mix was set up as follows:

ddH_2O	57.5μl
10x PCR Reaction Buffer (Gibco)	10μl
25mM $MgCl_2$	10μl (final 2.5mM)
2mM dNTPs (Pharmacia)	10μl
DNA template	2μl
forward primer (20μM)	5μl
reverse template (20μM)	5μl
Taq DNA polymerase (10U/μl,Gibco)	0.5μl

The mixture was mixed thoroughly and 50μl mineral oil added to prevent evaporation. The reactions were carried out in a Perkin-Elmer Thermal Cycler 480 with the following program:

Program segments	Temperature	Time	Cycles
1	95°C	2min	1
2	95°C 55°C 72°C	1min 1.5min 2min	25-30
3	72°C	10min	1
4	4°C	hold	-

The products were electrophoresed on an agarose gel (see section 5.3.3.2) and the appropriate sized fragments were purified using the Pharmacia Sephacryl HR resin S-400 Microspin columns (see Section 4.11).

5.3.6.1 Design of oligonucleotides

Oligonucleotides used to amplify and later clone fragments of defensin promoter were based on the HNP-3 defensin 5' upstream sequence cloned in our group (364) and of the near-identical published upstream sequence of the HNP-1 defensin promoter (45). HindIII and BamHI endonuclease restriction sites were included into the 5' extremities of the forward and reverse primers respectively, allowing cloning into the growth hormone vector. The forward and reverse primers used for amplifying the growth hormone cDNA for later cloning and expression as the reporter gene in our reporter vectors, are shown below. The former contains a Kozak sequence between the restriction enzyme site 5' and the specific growth hormone sequence 3' for good hybridisation. Similarly, the reverse primer has an artificial polyadenylation sequence added between the restriction enzyme site 5' and the specific growth hormone sequence 3'. These are shown below:

Forward primer: 5' ATTCAAGGATCCAGCCACCTCTAGACTGCCATGG 3'

Reverse primer:5'TAGTCGAATTCTTAATTTTATTGATCAGCTAGAAGCCAACAGCTGCCC 3'

The *cd18* promoter sequence cloned into the growth hormone reporter gene was synthesised chemically in order to have the right restriction enzyme sites at the ends of the sequence. The sense strand of that sequence is noted below:

5' GCTTCCACTTCCTCCAAGGAGGAGCTGAGAGGAACAGGAAGTGTCAGGACTTTACGACC
CGCGCCTCCAGCTGAGGTTTCTAGAG 3'

5.3.6.2 Synthesis of oligonucleotides

Oligonucleotides were synthesised on an Applied Biosystems model 381A DNA synthesiser according to manufacturers' instructions. 5' trityl groups were removed by the machine and the DNA immobilised on a column.

5.3.6.3 Purification of oligonucleotides

The DNA was eluted in 29% (v/v) ammonia by passing the solution through the column once every 5min for 1h. This solution was sealed in a glass vial and incubated at 55°C overnight to deprotect the oligonucleotides. Oligonucleotides were precipitated by adding 1ml of butan-1-ol to 100-150μl of the ammonia stock, the mixture incubated at 20°C for 10-15min and the oligonucleotides pelleted for 5-10min at 14,000rpm in an Eppendorf microfuge. The supernatant was discarded and the butanol was removed by a speedivac. The dried pellets were resuspended in 100μl of 1xTE buffer (see section 5.2.2). Oligonucleotides were quantitated as described in section 5.3.4 and a 20μM dilution prepared for use in PCR. Oligonucleotides which were used for making EMSA probes were firstly purified as above and then diluted to the required concentration and hybridised as described in section 5.3.9.1.

5.3.6.4 Site directed mutagenesis by PCR

Site-specific mutations were introduced using a method previously described by S.F. Michael (361) . Briefly, a thermostable ligase is inserted into the polymerase chain reaction to incorporate a phosphorylated mutagenic oligonucleotide primer into the amplified product. The mutagenic oligonucleotide was phosphorylated using the T4 polynucleotide kinase from Gibco BRL in the reaction indicated below :-

10x kinase buffer	5μl
100mM spermidine	5μl
10mM [^{32}P]-dATP	5μl
T4 polynucleotide kinase	4μl
Oligonucleotide	15μg
Water	up to 50μl

This was incubated for 1.5 hours at 37°C. Then 100μl of TE (see section 5.2.2) was added, and the aqueous phase extracted, and the DNA precipitated after addition of 0.1 volumes of 0.3M sodium acetate and 2.5 volumes of 100% ethanol overnight at –20°C. The precipitated oligonucleotide was then pelletted by centrifugation washed in 70% ethanol at –20°C, air dried and resuspended in TE. The purified oligonucleotide was quantified by spectroscopy and a 20μM dilution prepared. PCR amplification was then performed with the following reaction contents, a modified reaction mix from that presented in the original paper :-

ddH_2O	42.25µl
10x PCR Reaction Buffer (Gibco)	10µl
25mM $MgCl_2$	6.25µl
2mM dNTPs (Pharmacia)	16µl
DNA template	10µl
forward primer (20µM)	1µl
reverse template (20µM)	1µl
mutagenic primer (20µM)	2µl
Taq Ligase buffer	10µl
Taq Ligase	1µl
Taq DNA polymerase (10U/µl,Gibco)	0.5µl

The mixture was mixed thoroughly and 50µl mineral oil added to prevent evaporation. The reactions were carried out in a Perkin-Elmer Thermal Cycler 480 with the following program:

Program segments	Temperature	Time	Cycles
1	93°C 55°C 73°C	5min 5min 15min	1
2	93°C 55°C 73°C	1min 1.5min 2min	25-30
3	73°C	15min	1
4	4°C	hold	-

When assessed by agarose gel electrophoresis, only one PCR product was observed, though the original paper describes other products being formed between the mutagenic oligonucleotide and the reverse oligonucleotide. No such product was detected in these experiments. The PCR product was cloned into the reporter vector and DNA from different clones selected by antibiotic resistance; it was sequenced by the automatic sequencer to check whether the required mutation had been incorporated into the DNA.

5.3.7 Northern Blot Analysis

5.3.7.1 Isolation of cellular RNA

Total cellular RNA was prepared using the TRIzol method (Gibco), following the manufacturer's instructions. Cells were grown and harvested by centrifugation at 1,200rpm for 5min in a MSE Centaur benchtop centrifuge. After removed of growth medium, the cells were lysed by dispersing the cell pellets in TRIzol Reagent at a density of 5-10x10^6 cells per ml followed by incubation at 20°C for 5min. At certain differentiation time points, cells adherent to the plastic bottoms of the flasks were present in considerable numbers (such as in the later stages of monocytic differentiation). In these cases, Trizol mixture (or part of the total volume to be used) was directly applied to the flask after having harvested floating cells and medium so as to lyse the cells in situ. This TRIzol was then aspirated and added to that added to the pelleted suspension cells. Then 0.2ml of chloroform per 1ml of TRIzol was added and samples vortexed vigorously for 15sec and the incubation continued at room temperature for 2-3min. Samples were then centrifuged at 14,000rpm in an Eppendorf

microfuge at 4°C for 15min and the upper colourless aqueous phase containing RNA was transferrred to a fresh tube. The RNA was precipitated by incubating with 0.5ml of isopropanol per ml of TRIzol Reagent at RT for 10min. Samples were then centrifuged as above for 10min and RNA pellets washed with ice-cold 75% ethanol using at least 1ml of 75% (v/v) ethanol per ml of TRIzol Reagent. RNA was spun down as above, the pellets air dried for 5min and dissolved in a buffer containing 10mM EDTA (pH8.0), 1% (w/v) SDS and 1μg/μl proteinase K. To facilitate dissolving, RNA samples were incubated at 55-60°C for 5-10min.

To avoid degradation by contaminating RNases, a number of precautionary steps were taken. First, plasticware rather than glassware was preferred for handling and storage of solutions. All tubes were autoclaved before use. Second, all solutions including ddH_2O were pre-treated with 0.1% (v/v) DEPC, an irreversible inhibitor of RNases, and autoclaved. Treatment with DEPC was carried out in a fume hood for 3h at 37°C or overnight at room temperature. Third, the buffer used to dissolve RNA contained SDS and proteinase K and this serves to inhibit any residual RNases or those that carried over during subsequent handling of the samples. Finally, all solutions were pre-cooled in ice and all manipulations and centrifugations were carried out at 4°C. The concentration was determined spectrometrically (see section 5.3.4)

5.3.7.2 Electrophoresis of RNA

20μg of RNA was ethanol precipitated (see section 5.3.2.1) and the pellets resuspended in 22μl of RNA sample buffer freshly made as below. Formaldehyde gel-running buffer is (5x FGRB: 0.1M MOPS [pH7.0], 40mM NaAcetate and 5mM EDTA [pH8.0]).

10x formaldehyde gel-loading buffer is (10x FGLB: 0.25% (w/v) bromophenol blue, 50% (v/v) glycerol and 1mM EDTA [pH8.0]).

2μl	5x formaldehyde gel-running buffer
3.5μl	formaldehyde (40.6%, v/v)
10μl	formamide (>99%)
2μl	10x formaldehyde gel-loading buffer
2.5μl	ddH_2O
2μl	Ethidium bromide (10μg/ml)

The RNA samples, with 5μg of RNA size markers analysed in parallel, were denatured by incubation at 65°C for 15min followed by immediate chilling on ice. They were then loaded in 1.1% (w/v) agarose gel containing 6.5% (v/v) formaldehyde in 1xFGRB and electrophoresis was carried out in 1xFGRB (see above) at 80mM for 3-4h. At the end of electrophoresis, RNA gels were visualised under a TF-35M UV transilluminator and 28s, 18s and low molecular weight RNA bands seen could give a rough idea with regard to the quality of RNA samples and also act as a loading control.

5.3.7.3 Transfer of RNA to membrane

Following electrophoresis, RNA gels were rinsed in dH_2O for 5min, followed by soaking in 20xSSC (3M NaCl, 0.6M sodium citrate, pH7.0) for 20-60min. They were then transferred to Hybond N nylon membrane (Fingerprint grade) by capillary action overnight in 20 x SSC using multiple layers of tissue paper pressed down by a weighted glass plate. After transfer, the membrane was air-dried and UV cross-linked

using a UV Stratalinker 1800 UV cross-linker. Lanes containing RNA markers were fixed in 0.5% acetic acid for 5-10min and then stained with 0.04% (w/v) methylene blue in 0.5M NaAcetate (pH5.2) for 5-10min to allow size identification for probed messenger RNAs.

5.3.7.4 Random-primed radiolabelling of DNA probes

All DNA probes used for hybridisation to Northern blots were labelled with [α^{32}P]dCTP using a random-priming kit (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, 100ng of probe in 8µl 1xTE (see section 5.2.2) was denatured by boiling at 100°C for 5min and chilling on ice immediately. To the denatured probe, the following were added:

10x hexanucleotide mix	2µl
0.5mM dNTPs (dATP+dGTP+dTTP)	3µl
[α-^{32}P]dCTP (10(Ci/µl)	5µl
Klenow enzyme (10U/µl, Gibco)	2µl

The reaction mix was incubated at 37°C for 30min. Unincorporated nucleotides were removed by gel filtration on Nick column (Pharmacia). To prepare the columns, the storage buffer was poured off, the column was filled with elution buffer (0.1xSSC, 0.1% SDS) and it was allowed to empty under gravitational flow. The probe was then loaded carefully onto the top of the column followed by 400µl of elution buffer. Any eluted liquid at this point was discarded. Another 400µl was added onto the top of the

column and the eluent was now collected. This eluent containing the radio-labelled probe was boiled for 5min and then chilled immediately on ice to denature the probe.

5.3.7.5 Pre-hybridisation and Hybridisation

The membranes were prehybridised in a 42°C shaking water bath for 1-3h or overnight depending on the strength of the signals expected. Hybridisation buffer contained 4xSSPE (20xSSPE: 3M NaCl, 0.2M NaH_2PO_4, 25mM EDTA, pH7.4), 50% (v/v) formamide, 5xDenharts (50xDenhardts: 1% (w/v) Ficoll-400, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) BSA), 0.5% (w/v) SDS and 200μg/ml denatured salmon sperm DNA. Following prehybridisation, radio-labelled probe was boiled for 5min, chilled on ice and added to the hybridisation buffer. The membrane was subsequently hybridised overnight under the conditions described above.

5.3.7.6 Washing and Autoradiography

After hybridisation, the membranes were washed under increasingly stringent conditions as follows: twice in 2xSSPE, 0.1% (w/v) SDS at 25°C for 10min, once in 1xSSPE, 0.1% (w/v) SDS at 25°C for 10min and finally 2-3 times in 0.1xSSPE, 0.1% (w/v) SDS at 65-68°C for 10-15min. The membranes were then exposed to a Kodak X-OMAT-AR imaging film at -70°C for the required time.

5.3.7.7 Stripping of blots

The radio-labelled probe was stripped from the blots by placing in boiling 0.1% SDS and shaking until the solution cooled to room temperature. The blots were then hybridised to another probe as described above.

5.3.8 DNAse 1 Footprinting

DNase 1 footprinting analysis was carried with slight modifications to the method described by Plumb and Goodwin (365).

5.3.8.1 Generation of radiolabelled probes

50μg of plasmid DNA was digested with an appropriate restriction enzyme in a total volume of 100μl for 2-3h (see section 5.3.3.1). At the end of the digestion, 20μl of calf intestinal alkaline phosphatase (1U/μl, Boehringer Mannheim, diluted from purchased stock) was added and the incubation continued at 37°C for another 30min. Then, 10μl of 10% (w/v) SDS, 5μl of 2M NaCl and 5μl of EDTA (pH7.5) was added and the mixture heated at 68°C for 5min to inactivate the enzyme. The DNA was Phenol-Chloroform-Isoamyl alcohol extracted and ethanol precipitated (see section 5.3.3.1) . The pellets were dissolved in 45μl of a solution containing 100mM Tris.HCl and 1mM EDTA (pH8.0). 7μl of such dephosphorylated DNA (approximately 7μg) was used for each 5'-end labelling reaction as follows.

The following reaction mix was prepared:

dephosphorylated DNA (~1μg/μl)	7μl
10x Kinase Buffer (Gibco)	3μl
50mM DTT	3μl
20mM spermidine	3μl
ddH_2O	11μl

The mixture was heated at 70°C for 5min to denature the ends of the DNA and then quickly chilled at -20°C. It was then thawed on ice and 5μl of [γ-^{32}P]dATP (10μCi/μl), Amersham) and 0.7μl of T4 polynucleotide kinase (10U/μl, Gibco) were added and incubated at 37°C for 1h before adding 1μl of 0.2M EDTA (pH7.5) and incubation at 70°C for 10min to inactivate the enzyme. Then 100μl of TE (see section 5.2.2) was added and the mixture deproteinised by PCI extraction (see section 5.3.2.1). The aqueous phase was transferred to a fresh microfuge tube and 10μl of 4M NaCl, 3μl of calf liver tRNA (10mg/ml, Boehringer Mannheim) added, and the DNA was precipitated with 3 volumes of ethanol (see section 5.3.2.1). The pellets were dissolved in 90μl of TE (see section 5.2.2), 10μl of 1M KAcetate added and the DNA was re-precipitated with ethanol as above. The DNA pellets were then dissolved in 17μl of ddH_2O and the second restriction enzyme digest carried out in the appropriate buffer in a total volume of 20μl for 2h. At the end of the incubation, 5μl of 6x DNA gel-loading buffer (see section 5.3.3.1) was added and the 5' labelled probe separated from the rest of the plasmid by gel electrophoresis using a 1-1.5% agarose gel (see section 5.3.3.2). The gel was exposed to a Kodak X-OMAT-AR imaging film for 5min, the film developed and the radiolabelled DNA excised from the gel by aligning the autoradiograph with the gel. A second exposure

was then taken to ensure this had been adequately removed. The probe was then purified using the QIAquick Gel Extraction Kit for Agarose gel (see section 5.3.3.3) and the DNA was eluted into 100µl of ddH_2O.

5.3.8.2 Isolation of nuclear protein

NB4, HL60 or HeLa cells were cultured as described (see section 5.1.1) and eventually transferred to Roller bottles (Falcon, Becton Dickinson) incubated on a New Brunswick RollaCell roller at 37°C to increase the amount of cultured cells. When the cell number had reached approximately 10^9, they were pelleted by spinning at 1700 rpm (860g) for 10min at 4°C in a H6000A/HBB6 swing-out rotor using the Sorvall centrifuge RC3C. The pellets were washed once with ice-cold TMS solution (5mM Tris.HCl, 2.5mM $MgCl_2$ and 125mM sucrose, pH7.5). The volume of the pellet was estimated and this was resuspended in 4 times its volume of 2x TMS with Triton X-100 (about 0.1%), 1mM DTT, 10mM β-mercaptoethanol and proteinase inhibitors and incubated on ice for 10 minutes. (The exact percentage of Triton -X-100 was adjusted for different states of NB4 differentiation and for the different cells types to make sure there was cytoplast rupture but that nuclei remained intact after homogenisation, as judged by phase-contrast microscopy).

The freshly added proteinase inhibitors (0.5mM benzamidine, 10mM β-glycerophosphate, 2mM levamisole, 0.5mM PMSF, 10mM sodium butyrate, 5mM sodium orthovanadate (pH8.0) and 1µg/ml each of aprotinin, bestatin, leupeptin and pepstatin), 1mM DTT and 10mM β-mercaptoethanol were also added to TMS solutions used in all the following steps. The cell suspension was homogenised with 30-40

strokes of a Dounce homogeniser and centrifuged at 2000 x g for 10 minutes at 4°C. The pellet was washed first in 2xTMS with Triton (at the same concentration as before), and then in 2xTMS. The pellet was suspended in 10ml of 2xTMS and nuclear integrity assessed by microscopy. 750µl of 5M NaCl was added drop-wise on ice and the solution incubated for 30 minutes with continuous stirring. The suspension was centrifuged at 2000g for 10 minutes at 4°C, and the supernatant kept. The pellet was resuspended in half the original volume, and the addition of NaCl and centrifugation repeated. The pooled supernatants were centrifuged in a T1270 rotor using Sorvall OTD- ComBI Ultracentrifuge at 35,000rpm for 1h at 4°C. The supernatant was removed, its volume measured, and 0.45g $(NH_4)_2SO_4$ added per ml. The mixture was vortexed to dissolve the salt and then stirred on ice for 30min. The suspension was centrifuged at 15,000 rpm for 15 minutes at 4°C using the Sorvall RC-5B Superspeed Centrifuge. The supernatant was discarded and the pellet resuspended in 2ml of 2xTMS containing 0.35M NaCl. This was dialysed overnight against 1 litre of protein storage buffer (50mM NaCl, 20mM Hepes, pH 7.9, 5mM $MgCl_2$, 1mM DTT, proteinase inhibitors and glycerol 20% v/v). The next day, the samples were ultracentrifuged in a TLA100.3 rotor using the Beckman centrifuge TL100 at 35,000rpm for 1h at 4°C. The supernatant was aliquotted into sterile microfuge tubes, snap frozen in liquid N_2 and stored at -70°C.

5.3.8.3 G and A tracking reactions

The positions of footprints within the promoter were localised by utilising A+G chemical sequencing reactions (366). 9.5µl of ^{32}P-labelled probe were mixed with 0.5µl of yeast tRNA (10mg/ml) and 1.5µl of 88% (v/v) formic acid. The reaction mix was incubated at 37°C for 14min, chilled on ice and 150µl of freshly prepared 1M aqueous

piperidine added to cleave the DNA at 90°C for 30min. At the end of the incubation, the samples were chilled on ice and precipitated by the addition of 1ml of butan-1-ol followed by spinning at full speed in a microfuge for 2-5min. The pellets were resuspended in 1% SDS and re-precipitated with 1ml of butan-1-ol as above. The pellets were dried in a Heto Vac speedivac (Inter Med) for 5min, resuspended in 10-30μl of 1x Sequencing Gel Loading Buffer (see section 5.3.8.4) and then separated in a urea denaturing polyacrylamide (see section 5.3.8.4) gel along with samples from footprinting reactions.

5.3.8.4 Footprinting reaction

Nuclear protein extract (40μg or 200μg) was incubated with approximately 30ng of (-240/+15) *defensin* promoter sequence ($1x10^3$ cpm/ng), end-labelled with $\gamma^{32}P$-dATP on one strand and 6μg of poly (dIdC) on ice for 1 hour in a total volume of 100μl of protein storage buffer (with proteinase inhibitors as indicated in section 5.3.8.1). Incubation was then further continued for 20 minutes at 25°C. In the control reaction bovine serum albumen was used instead of nuclear proteins.

As DNA fragments are extremely sensitive to DNase1, minor inconsistencies in handling could cause large differences in digestion. To circumvent this problem: handling was kept as consistent as possible; the reactions were carried out one by one and, in addition various amounts of DNase I were used in each experiment. Briefly 0.5, 1.0 or 1.5 U of DNAaseI (Boehringer Manheim - diluted from purchased stock to 1U/μl) was added to the protein-DNA incubation, the mixture briefly vortexed and then incubated for 20 seconds at 25°C. Reactions were stopped by adding, footprint stop buffer (resulting in a final concentration in the reaction tube of 4 x TE (pH8), 0.5%

SDS, 0.5M NaC1, 0.33μg/ml tRNA, and 2μg/ml proteinase K) and transferring the sample onto ice. The mixture was incubated at 37°C for 30 minutes to allow protein digestion by the proteinase, and the DNA denatured at 90°C for 2 minutes. The DNA was then deproteinised by two phenol/chloroform/isoamyl alcohol extractions and a chloroform extraction. Nucleic acid was ethanol precipitated overnight using 15μl of 5M LiCl (final concentration, 0.4M) and by the addition of 3 volumes of ethanol. The DNA was pelleted by spinning the samples at 14,000rpm in an Eppendorf microfuge and the pellets were washed once with ice-cold 75% (v/v) ethanol. After air-drying, the pellets were dissolved in 8μl of sequencing buffer (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% Xylene Cyanol FF).

5.3.8.5 Gel analysis

The DNAse 1 digested and deproteinised DNA in sequencing buffer was denatured for 3 minutes at 90°C, cooled on ice and 4μl of the sample was loaded onto a denaturing (42% w/v urea) 6% polyacrylaminde gel which had been pre-run for 1 hour at 66 watts (1.6kV) at room temperature with 1xTBE as running buffer. After sample loading, the gel was run in similar conditions for 2-3 hours, vacuum dried on Whatman paper, and exposed to Kodak imaging film. The polyacrylamide gels were prepared from a stock solution with 40% acrylamide and 2.1% bis-acrylamide as described in Sambrook et al (357).

5.3.9 Electrophoretic Mobility Shift Assay(EMSA)

5.3.9.1 Preparing double stranded oligonucleotides

250pmols of each complementary oligonucleotide were added to a solution containing 0.1M NaCl in 1xTE (see section 5.2.2) in a total volume of 100μl. The mixture was boiled in a water bath for 10min and then left to slowly cool down to RT in the water bath. The concentration of the annealed double-stranded oligonucleotides was 2.5pmols/μl. Oligonucleotide probes used for band shifts are shown below (references from where competitor oligonucleotides were obtained are also indicated).

FPα: ACAGAAAGTAACCCCGGAAATTAGGACACCTCATCCC

FPα mut-ets: ACAGAAAGTAACCCCactAATTAGGACACCTCATCCC

FPβ: TTTAACCTCACCTTCCCACCAAATTTCTCAACTGTCCTTGCCACCACA

FPβmut-c/ebp: TTTAACCTCACCTTCCCACCAAgggTaTCAACTGTCCTTGCCACCACA

FPβmut-myb: TTTAACCTCACCTTCCCACCAAATTTCTCgAgTcTCCTTGCCACCACA

FPβmut-aml: TTTAAtCTCgCCTTCCCACCAAATTTCTCAACTGTCCTTGCCACCACA

FPγ: GGTAGATGAGAGGTTCCTCTGTGGAGTTCTACTTTAA

FPγ mut-ets: GGTAGATGAGAGGTagtTCTGTGGAGTTCTACTTTAA

FPγ mut-pu.1: GGTAGATGAGAGGTTCCgaTGTGGAGTTCTACTTTAA

Oligonucleotide name	Oligonucleotide sequence	Reference
ets (*neutrophil elastase*)	GTGTCCCCAGGGAGGAAGTAGGGCT	(321)
pu.1 (*cd11b*)	CTTCTGCCTCCTACTTCTCCTTTTCTGCCCT	(304)
myb (SV40 enhancer)	TTCGGCATAACGGTTCCGTAGCC	(367)
c/ebp (*MIP-1α*)	GCTGCAGATTGCGCAATCTGCAGC	(263)
c/ebp-aml(*M-CSF Receptor*)	CAAGATTTCCAAACTCTGTGGTTGCCTTGC	(238)
aml (*neutrophil elastase*)	CAGTAGGGCTGTGGCCAGGATGGG	(354)
mut-aml (*neutrophil elastase*)	CAGTAGGGCcGaGaCCAGGATGGGG	(354)

5.3.9.2 Radiolabelling of probes

5'-end labelling of double stranded oligonucleotides was carried out as previously described by Plumb *et al* (368). 2μl (5pmols ~ 200ng) of double-stranded oligonucleotide annealed as described above was 5'-[^{32}P]-end labelled in the following reaction:

1μl	10x kinase buffer (Gibco)
0.5μl	100mM DTT
1μl	20mM spermidine (Sigma)
2.5μl	ddH_2O
2μl	[γ-^{32}P] dATP (10μCi/μl, Amersham)
1μl	T4 polynucleotide kinase (10U/μl, Gibco)

The above mixture was incubated at 37°C for 45minutes. The labelled oligonucleotides resulting from this reaction were then electrophoresed in a 1xTBE (see section 5.2.2), 8% (w/v) polyacrylamide gel (prepared from a stock with 40% acrylamide and 2.1% bis-acrylamide) at 100-150V for 1-2h in 1xTBE running buffer. The gel was exposed to a Kodak X-OMAT-AR imaging film for 2min, the film developed and the radiolabelled oligonucleotides excised from the gel by aligning the autoradiograph with the gel. A second exposure was then taken to ensure the oligonucleotides had been removed. The gel slice was pureed by centrifuging it through a pinhole in the bottom of a 250μl microfuge tube into a 500μl tube and this then incubated at 37°C overnight in 400μl 1xTE (see section 5.2.2). The gel fragments are then pelletted by rapid centrifugation in an Eppendorf microcentrifuge and the supernatant is used as the probe for EMSA experiments. To estimate the amount of unlabelled sequence used in competition experiments it was assumed that

80% of the oligonucleotides was recovered during the procedure, giving a concentration of labelled oligonucleotides of approximately 5 fmols/μl.

5.3.9.3 Isolation of nuclear protein

NB4 Nuclear protein extract used for EMSA was the same extract that was used for footprinting reactions, the rationale being that similar features of transcription factor-DNA binding were being studied in both experimental procedures so it did not make sense to use a different type of protein extract. HL60 or HeLa proteins extracts were prepared in a similar manner except for that used in the figure 34a where the extract used was produced by a rapid micropreparation technique exactly as described by Andrews and Faller (369).

5.3.9.4 Incubation reaction and gel analysis

Unless otherwise specified, nuclear protein extract (5μg) was pre-incubated with unlabelled DNA sequences (including 2 pmoles of competitor oligonucleotides where applicable) for 10 minutes on ice with 15μl of buffer 1 including proteinase inhibitors in final concentrations similar to those used in footprint protein storage buffer (see section 5.3.8.1) The final buffer concentration in the reaction was: 5% glycerol, 190mM KC1, 50mM Hepes, pH7.5, 0.3% non-fat milk, 0.25% Nonidet P-40, and 25μg/ml poly dIdC. 2μl of γ^{32}P-dATP end-labelled probe (approximately 10 fmol, 10^5 cpm) was then added up to a total volume of 20μl and the reaction incubated at 25°C for 20 minutes. DNA-protein complexes were separated by electrophoresis through a 6% non-denaturing polyacrylamide gel with 0.5 x TBE as running buffer (30v, 50mA) for

about 3.5 hours at 25°C after pre-running for about half an hour under the same conditions. Gels were fixed in 10% acetic acid/methanol, vacuum dried and exposed to photographic film (Kodak X-OMAT- AR5).

5.3.9.5 Modifications of EMSAs including supershift analysis

Modifications to the above procedure are described when used in particular experiments in the text and particularly in the figure legends. These include different incubation lengths, pre- or post-addition of antibodies, different electrophoresis running buffers and different gel concentrations to enhance separation of particular complexes. One common variable introduced was the use of different buffers with different reaction volumes. These different buffers were originally used in order to better replicate the conditions for particular antibody-antigens reactions as had been originally described by their developers. Later, however, they were also occasionally used, in order to try and better view certain particular complexes or interactions. These buffers were buffer 2 which was used by Chumakov et al. (370) with the anti-N-terminal C/EBPε antibody but modified with dIdC (1μg/reaction) as non-specific competitor instead of salmon sperm DNA. Generally, 5μg not 10 μg of nuclear protein extract was used in the reaction. The total reaction volume when using this buffer (final concentration 20mM Hepes, [pH 7.9], 20% glycerol, 125mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.1% Triton-X-100, 1mM DTT, 1mM PMSF, 10μg/ml leupeptin, 10μg/ml pepstatin A, and 100μg/ml aprotonin) was just 12 μl. When using this buffer, labelled probe was added immediately after nuclear extract (there was no pre-incubation between extract and unlabelled competitor DNA) and complexes formed were separated by electrophoresis through a 4.5 % (as opposed to 6%) polyacrylamide gel. Buffer 3 was used primarily

with antibodies to GABP (322). 10μg of nuclear protein extract (or 25 ng of purified GABP proteins) are used with this buffer (final concentration : 10mM Tris[pH 7.5], 50mM NaCl, 1mM EDTA, 1mM β-mercaptoethanol and 1% Ficoll) in a total reaction volume of 30μl, a modification from the 15μl total volume previously used). Poly dI-dC was added to the reaction in different concentrations depending on whether purified proteins (0.5μg) or nuclear extract (2.0 μg) were being analysed. Using this buffer, the whole incubation is just kept on ice for 10 minutes before electrophoresis through a 4.5 % polyacrylamide gel, more often than not with a 0.25 x TBE running buffer (which is better than the normal 0.5x TBE to allow GABP binding).

In "supershift" analysis, unless otherwise specified, 2μl of pre-immune serum or anti-serum were added to the DNA-protein binding mix just after addition of the labelled probe sequence. Incubation and gel electrophoresis were carried out as described (section 5.3.9.4).

5.3.10 Western Blot Analysis

5.3.10.1 Isolation of nuclear protein

Nuclear protein extract used in western blots for GABP and PU.1 detection were the same protein extract made for footprinting or EMSA both in the case of NB4 and in the case of HeLa nuclear extracts. Different time point nuclear protein made for Myb western blot analysis was made by rapid micropreparation as described by Andrews and Faller (369). Basically, 10^7 cells were pelleted by centrifugation at about 1500 rpm and resuspended in 1.5 ml of ice cold PBS. They were repelleted by centrifugation for 10seconds in an Eppendorf microfuge and the supernatant wash was discarded. The

pellet was resuspended in 400 μl hypotonic lysis buffer (10 mM Hepes/KOH [pH7.9], 1.5mM $MgCl_2$,10mM KCl, 0.5 mM DTT, 0.2 mM PMSF at final concentration) and left to swell on ice for 10 minutes. The suspension was then vortexed for 10 seconds to break the cytoplasmic membrane and release the nuclei. The nuclei were pelleted by microfuging for 10 seconds and the supernatant discarded. The pellet was suspended in about 50 μl of high salt buffer (20mM Hepes/KOH [pH7.9], 25% glycerol, 420mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2mM PMSF at final concentration) and incubated on ice for 20 minutes. The cellular and nuclear debris was pelleted in a microfuge through a 10 second spin and supernatant was split into 20μl aliquots, frozen in a dry-ice-alcohol bath and stored at -70°C.

5.3.10.2 Quantification of samples

The concentrations of protein extracts were determined by the Bicinchonininc acid protein assay. The working reagent was prepared by mixing 50 parts of reagent A (Bicinchonininc acid solution, Sigma) with 1 part of Reagent B ($CuSO_4.5H_20$ 4% w/v solution). 10μl of test protein samples or each of the BSA protein standards (80, 100, 200, 400, 1000 and 2000 μg/ml)was added to 200μl of the working reagent and the mixture was incubated at 37°C for 30min. The absorbance at 562nm was measured using a spectrophotometer.

5.3.10.3 SDS-Polyacrylamide gel analsis (PAGE)

For Western blot analysis, the appropriate amount of protein extract was mixed with an equal volume of 2x SDS loading buffer (100mM Tris –pH 6.8, 200mM DTT,

4% SDS, 0.2% bromophenol blue and 0.2% glycerol) and denatured by boiling for 5-10min. The samples were then ready for use in SDS polyacrylamide gel electrophoresis. Denatured protein samples in loading buffer were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the appropriate percentage (5-15%) of acrylamide resolving gels containing 0.1% (w/v) SDS, 0.1% (w/v) Ammonium persulphate(APS), 0.04% TEMED in 1.5M Tris.HCl (pH8.8). 5% acrylamide stacking gels, containing the same concentration of SDS, APS and TEMED, were made in 1.0M Tris.HCl (pH6.8). Gels were prepared from a stock with 30% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide. Electrophoresis was carried out in 1x Tris-glycine electrophoresis buffer (250mM glycine[pH8.3], 0.1% (w/v) SDS and 25mM Tris.) at 150-200V.

5.3.10.4 Wet Blotting

This procedure was carried out using the Bio-Rad wet transfer apparatus. Any unused parts of the gel as well as the stacking gel are cut away. 4 pieces of Whatman 3MM paper and 1 piece of Immobilon membrane, the size of the gel were cut out. 2 pieces of 3MM paper were soaked in Towbin buffer (4 litres are made with 12.11 g Tris, 57.6g Glycine, 800mls of methanol and 1.2 mls of concentrated HCl and water). These 2 soaked 3MM paper pieces are placed of one of the sponge sheets. Any air bubbles were squeezed out. The gel was washed in Towbin and placed on these 3MM paper pieces. The Immobilon was wetted in Towbin, placed carefully on the gel and extra care was taken to ensure no air bubbles were present between the gel and the membrane. The remaining two sheets of 3MM paper were similarly soaked in Towbin and placed on the nitrocellulose and again care was taken to ensure no bubbles were present. Any present were sqeezed out by rolling a glass rod over the sandwich. The remaining sponge sheet was placed over the whatman paper and the entire sandwich is

enclosed within the plastic holding frame. This sandwich plate was locked so as to hold the contents firmly in place between the sponge sheets and inserted into the tank compartment of the apparatus with the nitrocellulose on the side of the red plug (cathode) and the gel on the side of the black plug (anode). The tank was filled with Towbin, closed and transfer was performed over night at 4°C and 30 V.

5.3.10.5 Protein detection

The membranes were incubated in a blocking solution (5-10% (w/v) dairy milk powder('Marvel' see section 4.5) in 1x Tris-buffered saline-Tween (1xTBS-T: 20mM Tris, 137mM NaCl and 3.8mM HCl, pH7.6, plus 0.1% (v/v) Tween 20)) at 4°C overnight or at RT for 1-2h with constant vigorous shaking. After blocking, the blots were washed in 1xTBS-T (see above) as follows: two brief rinses at RT, one wash at RT for 15min with constant vigorous agitation and finally two washes at RT for 5min. Then the membranes were incubated with a 1:1-5,000 dilution of the primary antibody in TBS-T containing 5-10% (w/v) dairy milk power at RT for 1h or 4°C overnight with constant vigorous agitation. At the end of the incubation, the membranes were washed as described above and then incubated with a 1:5,000 secondary antibody in TBS-T containing 5-10% (w/v) dairy milk power at RT for 1h with constant agitation. The membranes were then washed again as described above and antibody binding detected using the ECL system (Amersham). Equal volumes of detection solution I and II were mixed and the mixture placed onto the membranes with protein-side up. After incubation for exactly 1min, the membranes were wrapped in Saran Wrap and exposed to a Fuji Medical X-ray imaging film for the appropriate length of time.

5.3.11 In Vitro Transcription/Translation

In vitro transcription and translation of the *Fli-1* protein were carried using the TNT T7 Quick Coupled Transcription/translation System (Promega) following the manufacture's instructions. The following reaction components were assembled:

TNT T7 Quick Master Mix	40μl
[^{35}S]methionine (10(Ci/μl, Amersham)	4μl
DNA template	1μg
nuclease-free ddH_2O	make up to 50μl

The reaction was incubated at 30°C for 90min. The radioactive translation products were analysed on an SDS-polyacrylamide gel as follows. 5μl aliquot was added to 20μl of 1x SDS-sample buffer (see section 5.3.10.3). After denaturation by boiling at 100°C for 2min, the samples were then analysed by SDS-PAGE (see section 5.3.10.3). At the end of the electrophoresis, the gel was dried down and exposed to a Kodak X-OMAT AR imaging film for 6-16h at RT. For producing non-radioactive translation products, 1μl of 1mM unlabelled methionine was substituted for the [^{35}S]methionine for gel retardation assays (see section 5.3.9.4).

Results

Chapter 6: Characterisation of the NB4 cells

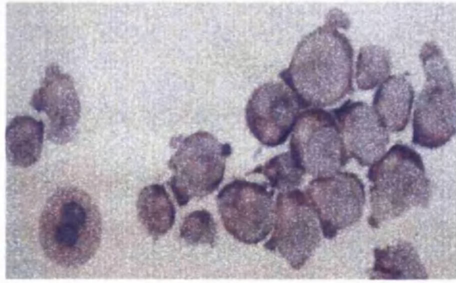
Having obtained a clone of the differentiable, NB4 acute promyelocytic leukaemia cell line, which had already been shown to express *defensin*, we characterised these cells as regards differentiation and as regards *defensin* expression to assess whether they would represent a good experimental model for promoter studies.

6.1 Differentiation of NB4 cells - Morphological Changes

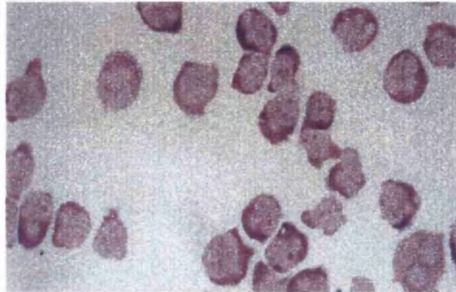
Undifferentiated NB4 cells appear as typical leukaemic blasts with large nuclear to cytoplasmic ratios and finely dispersed chromatin (Figs. 6 and 7). Nucleoli are not clearly defined and granules are also not identifiable, as is sometimes the case in Acute Promyelocytic Leukaemia.

During granulocytic differentiation, induced with 1 μM all-*trans* retinoic acid (ATRA), the nucleoli rapidly become prominent (by 24 hours) and the nuclear to cytoplasmic ratio begins to decline (Fig.6). Mitotic figures are no longer visible after 48 hours of ATRA-induction indicating a cessation of cell division. After 72 hours of granulocytic differentiation, the characteristic multi-lobed nucleus of the mature granulocyte begins to appear with the nuclear chromatin becoming particularly condensed by 5 days after the initiation of ATRA-induction. Concurrently the

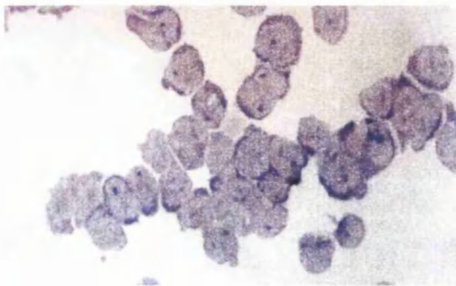
Fig.6 ATRA-induced maturation of NB4 cells towards the granulocyte neutrophil lineage.
Morphological evidence of maturation, as evidenced by May-Grunwald Giemsa staining. The panels to the left of the page show cells at a 400 x magnification whilst the panels to the right are magnified by 1000 x and viewed by oil immersion.



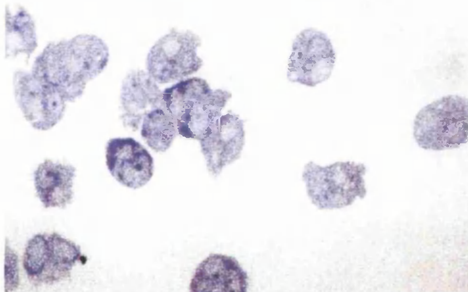
Undifferentiated NB4 cells



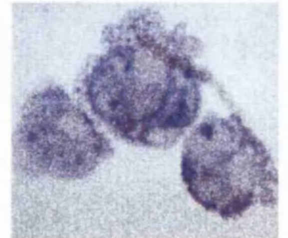
24 hours + ATRA

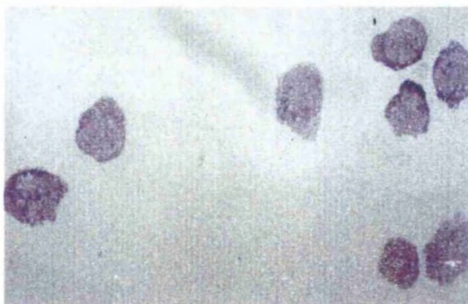


48 hours + ATRA

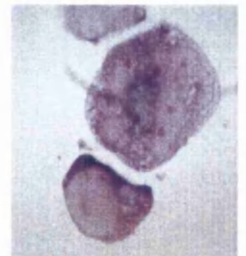


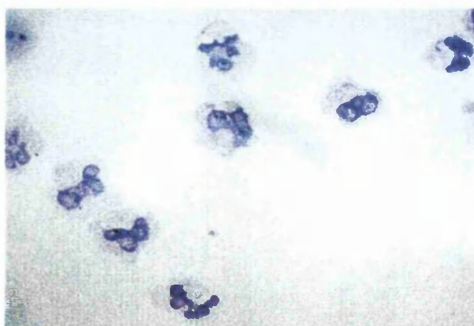
72 hours + ATRA





96 hours + ATRA





120 hours + ATRA

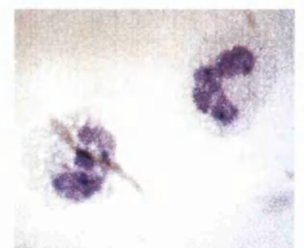
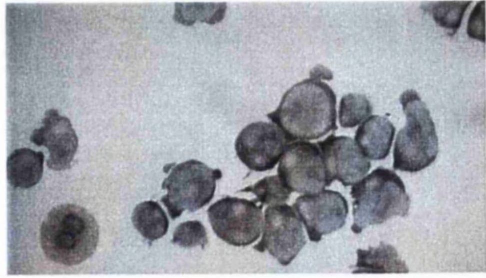


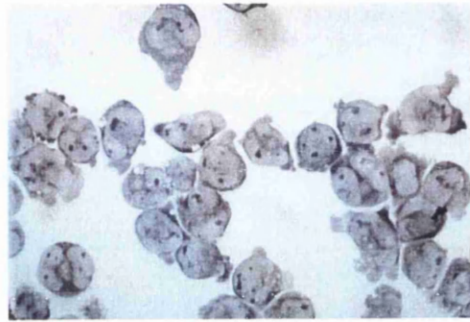
Fig.7 Vitamin D3 and Phorbol Myristate Acetate (PMA) - induced monocytic differentiation of NB4 cells.
Morphological maturation as evidenced by May-Grunwald Giemsa staining. The top three panels show cells at a 400 x magnification whilst the cells in the bottom panel are magnified by 1000 x and viewed by oil immersion.

NB4 cells
uninduced

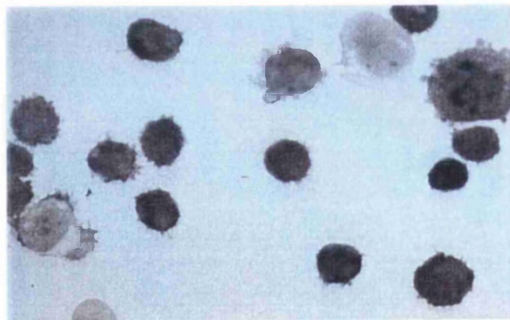


VitD3+TPA

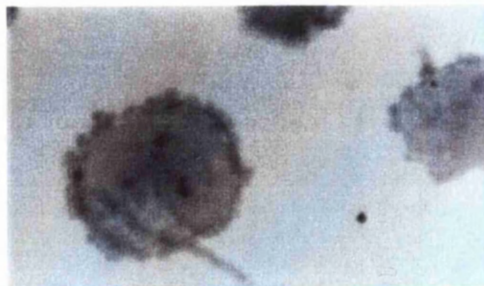
4 hours



24 hours



72 hours



cytoplasm becomes somewhat more granular and appears foamy probably due the presence of numerous vacuoles/granules.

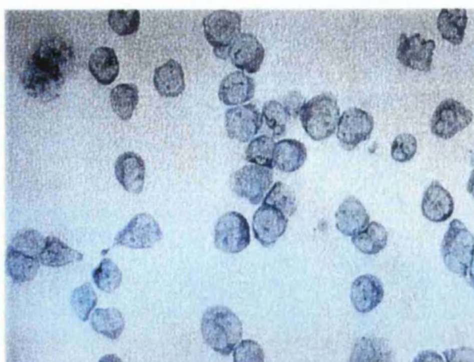
Monocytic differentiation is induced using 0.4μM Phorbol 12-Myristate 13-Acetate (PMA) and 0.4μM 1,25-dihydroxy Vitamin D3 (Fig.7). There is early withdrawal from the cell cycle as evidenced by the rapid disappearance of mitotic figures in less than 24 hours. By this time point a large majority of the cells (about 80%), are adherent to the plastic at the bottom of the flask. Nucleoli also become more prominent as during early granulocytic differentiation and small pseudopodia are identified as irregularities of the cellular circumference. These become more apparent as differentiation progresses resulting in a characteristic monocyte-like cell with horseshoe shaped nucleus, foamy cytoplasm and irregular cell outline by 72 hours of induction (Fig.7).

6.2 Functional Evidence of Granulocytic differentiation

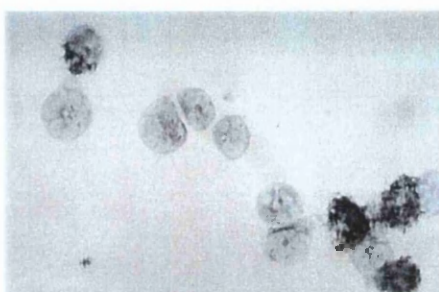
Granulocytic differentiation is monitored by the capability of NB4 cells to reduce the dye Nitroblue tetrazolium (NBTZ). The appearance of insoluble blue crystals within the cells allows the percentage of cells showing this activity to be counted (Fig. 8). By counting the number of blue cells and the total cell number within a fixed number of high power fields, the percentage of NBTZ-reducing cells at different time points of ATRA-induced differentiation can be plotted. Fig. 9 which shows a representative experiment indicates that at 48 hours of induction, 40 to 50 % of the cells are capable of converting NBTZ whilst by 96 hours, more than 80 % of the cells have acquired this potential. This variability in reduction activity is probably partly due to

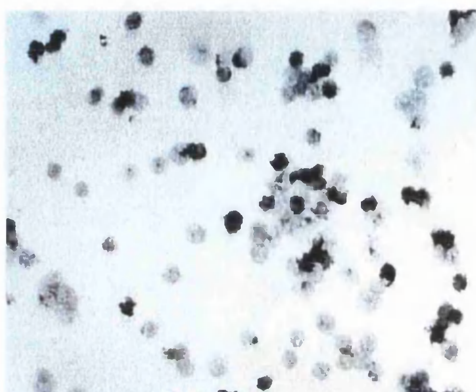
Fig.8 Changes in reducing ability during granulocytic NB4 cell differentiation. Nitroblue tetrazolium (NBTZ), a yellow dye is incubated with cells at different time points of differentiation. According to the reducing activity of the cells, the dye is converted into blue insoluble formezan precipitate. The left and topmost right panel show cells at a 400 x magnification whilst the cells in the 3 lower right panels are magnified by 100 x.

Undifferentiated NB4

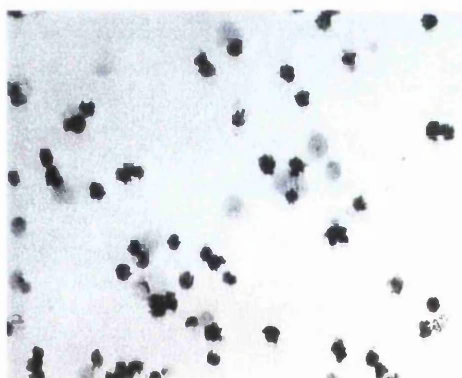


ATRA - 24 hours





ATRA - 48 hours



ATRA - 48 hours
(following transfection)

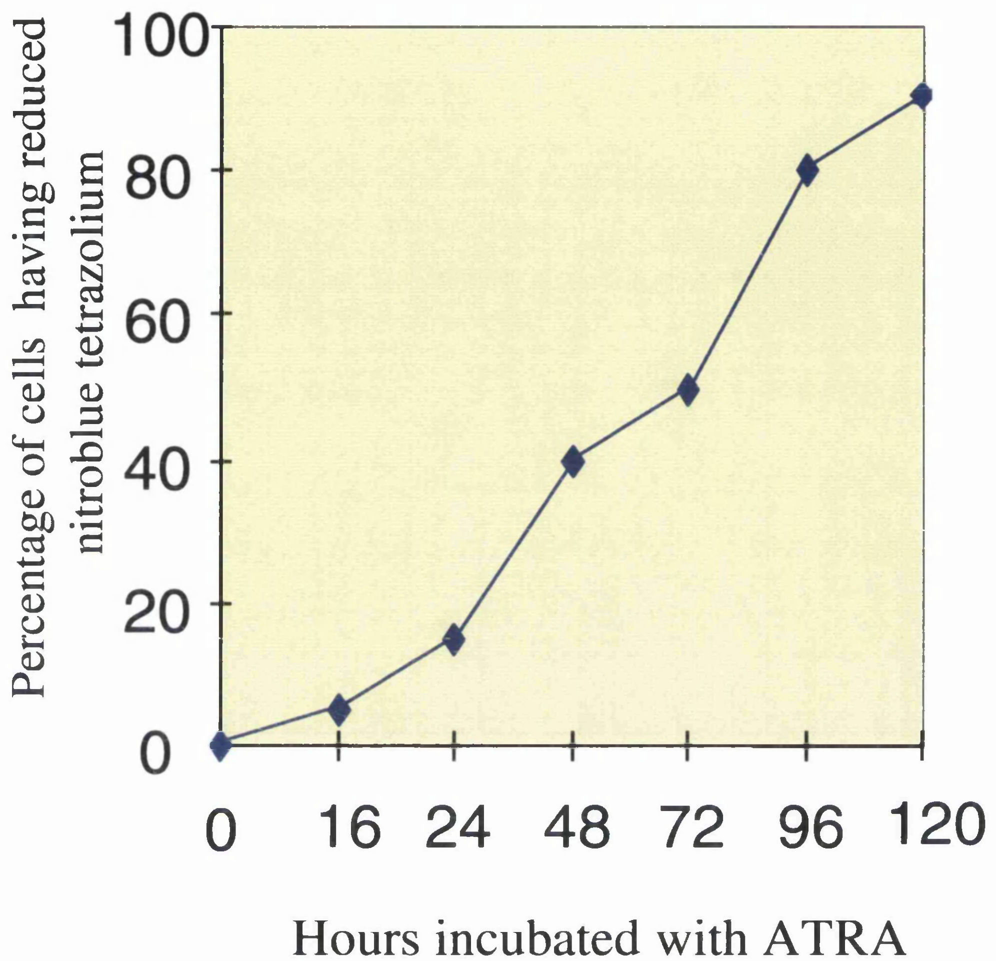


Fig.9 Assessing granulocytic differentiation by quantifying the amount of cells, reducing NBTZ.
Samples of the induced culture are assayed at different time points following the initiation of incubation with ATRA. The percentage of cells having such reducing ability is calculated from the fraction of blue-staining cells out of the total number of cells counted per high power microscope field. An average fraction calculated from 10 such fields is used.

cells being at different stages of the cell cycle within the growing NB4 population when induction was initiated. This may effect the actual initiation of differentiation in particular cells. Interestingly the cell concentration at induction of differentiation also influences the rate of differentiation (371) so uniformity between experiments is of the utmost importance. However studies with other myeloid cell lines have shown a certain cell-cycle independent variability since even if cells are sorted for cell cycle status and differentiated as a sorted population, some cells show activity as early as 24 hours after induction whilst the great majority do not (372). NB4 cells which are transfected after 20 hours of ATRA-induction and are assessed by NBTZ staining 48 hours after initiating induction, reduce the dye to a similar extent as do untransfected cells at this time point during differentiation (Fig. 8). This is very important and indicates that granulocytic differentiation is not significantly perturbed by the transfection procedure and that results obtained by these studies therefore accurately represent *defensin* promoter function in cells differentiated to a time point equivalent to non-transfected cells.

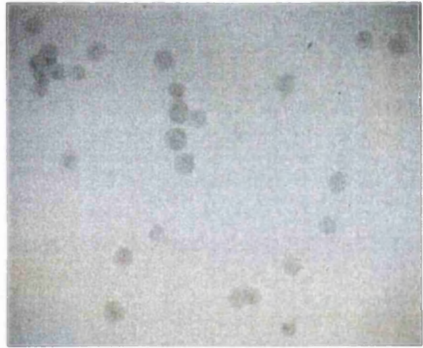
6.3 Functional Evidence of Monocytic Differentiation

Monocytic differentiation was assayed by non-specific esterase testing as well as by morphological changes. As can be seen in Fig. 10, a progressive increase in such esterase activity (as indicated by the dark intracellular staining) can be detected from as early as 4 hours after the induction of monocytic differentiation. The intensity of this staining within individual cells in the population is rather variable even at the later stages of differentiation where morphological transformation is clearly indicative of the

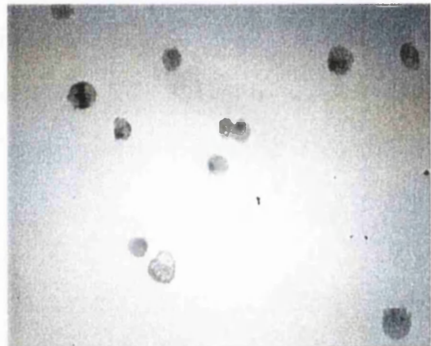
Fig.10 Non-specific esterase activity in NB4 cells induced to differentiate to monocytes.

Enzyme activity results in the conversion of a soluble dye to dark insoluble precipitates within the cells. The extent of activity clearly varies between different cells at the same time point following induction. The right panels show cells at a 100 x magnification whilst the cells in the lower left panels are magnified by 1000 x and is viewed by oil immersion.

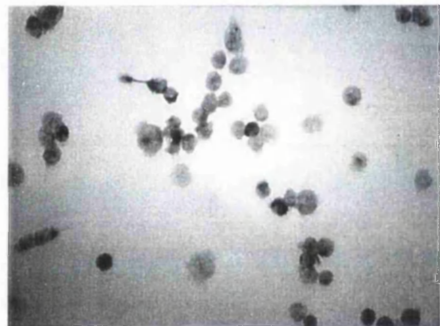
NB4 Undifferentiated



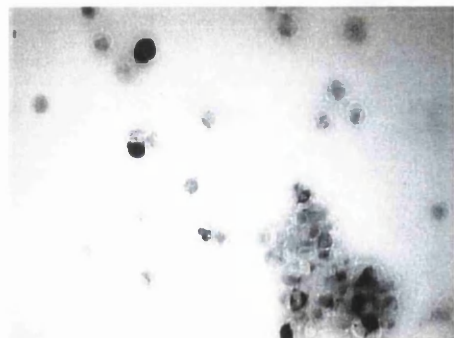
TPA+Vit.D3 - 4 Hours



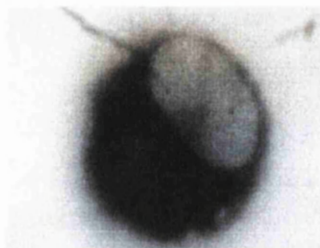
TPA+Vit.D3 - 24 Hours

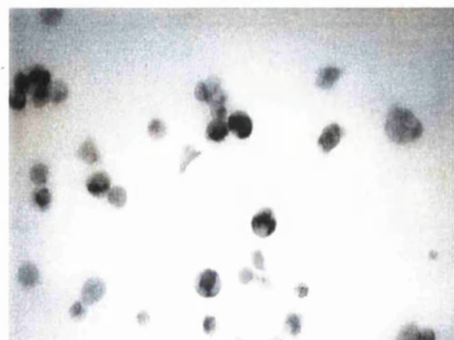


TPA+Vit.D3 - 48 Hours



TPA+Vit.D3 - 72 Hours





monocytic/macrophage phenotype. However, when compared to uninduced cells which lack any such staining, and counting the number of staining cells and total cells per high power field, the percentage of staining cells (albeit weak staining in many cells) increases rapidly to around 80% within the first 24 hours. After this there is a further small increase in staining as is shown in Fig.11 which documents a representative time course experiment. This pattern of rapid increase in esterase activity closely follows the rapid morphological changes seen during monocytic differentiation, as opposed to the more drawn out changes in granulocytic differentiation. The high power view of a heavily stained cell at 72 hours of differentiation clearly also shows the characteristic monocyte bi-lobed nucleus (Fig. 10)

6.4 *Defensin* expression during NB4 cell differentiation

Since studying the factors responsible for stage-specific expression was the aim of this work, *defensin mRNA* abundance in NB4 cells during chemically induced differentiation down each pathway was analysed. Northern Blots of RNA prepared at different time points during differentiation were probed with a complete *HNP-3 defensin* cDNA (Fig.12). The same blots were also probed with a β_2-*microglobulin* cDNA to control for loading differences between lanes.

During granulocytic (ATRA-induced) differentiation, there is a clear increase in *defensin* message abundance first detected at 16 hours post-induction and increasing to peak at the 24 and 48 hour time points. Studies of *defensin* expression upon chemical induction in HL60 cells indicate that the increase in mRNA abundance during ATRA-induced differentiation occurs as a result have increased transcription, as indicated by run-on assays (52). Whilst an increase in transcription is most probably responsible for

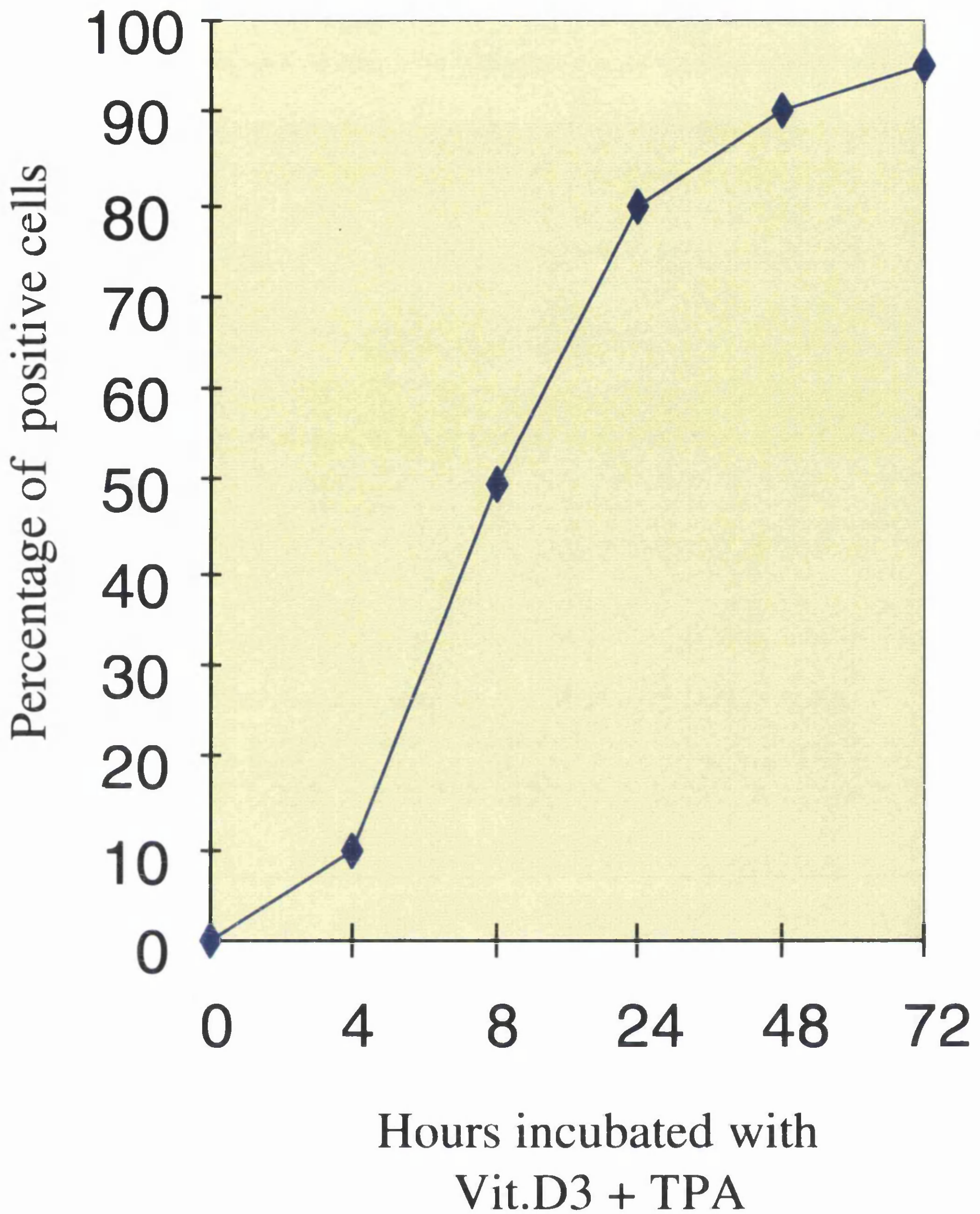
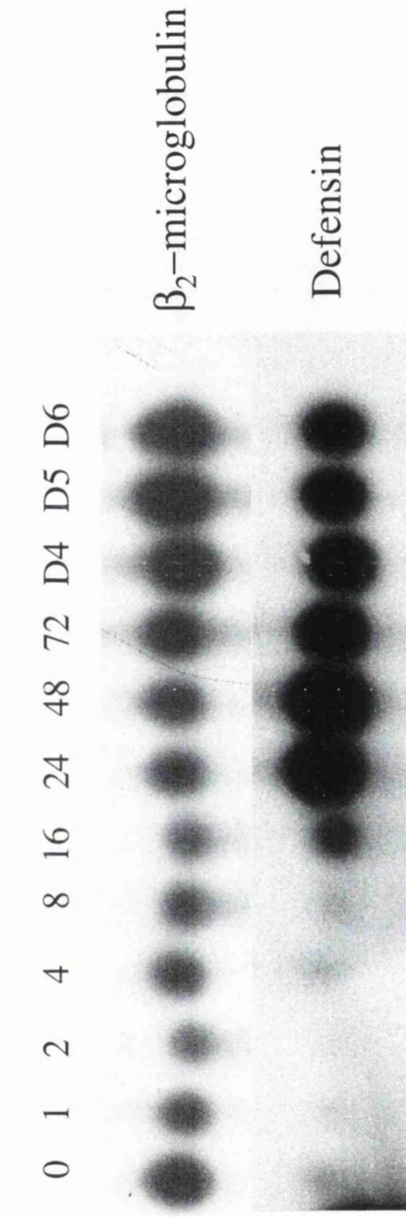


Fig.11 Measuring monocytic differentiation by quantification of the percentage of esterase-positive cells.
The percentage was calculated by identifying the fraction of cells darker than similarly stained uninduced cells, out of the total cells viewed per high power field. An average fraction from 10 such fields is used. Esterase activity is seen to rise sharply within a few hours of starting chemical induction after which a plateau is reached.

Fig.12 Northern blots of total RNA during chemically-induced granulocytic and monocytic NB4 cell differentiation.
The blots were probed with a radiolabeled defensin HNP-3 cDNA fragment (shown in the lower panel in each case). The same blot was re-probed with a labelled β_2-microglobulin cDNA as a loading control (shown in the upper panel). The difference in the strength of the defensin signal at 0 hours between the granulocytic and monocytic blots is due to the much shortened exposure in the granulocytic blot in order to prevent over-exposure of the signal at later time points.

a



b

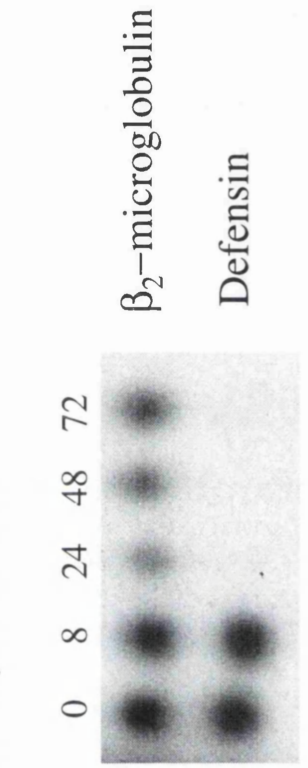
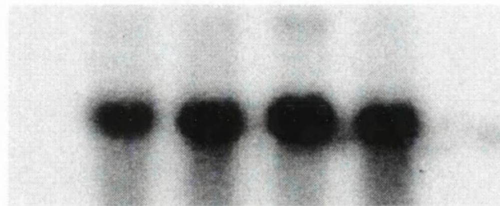


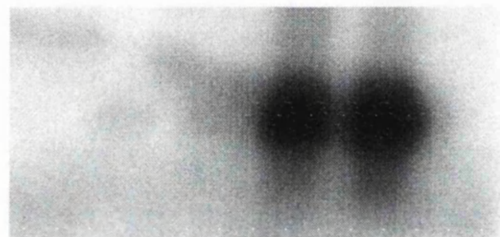
Fig.13 Increased defensin mRNA accumulation with differentiation.
Northern blot of total RNA from different time points during ATRA-induced granulocytic differentiation of NB4 cells was prepared. The blot was probed with a labelled b-actin cDNA (top panel) and subsequently with a labelled defensin HNP-3 cDNA fragment (middle panel). Ethidium bromide staining of the gel, showing the 18S-rRNA band was used as the loading control (lower panel).

Hours of ATRA induction

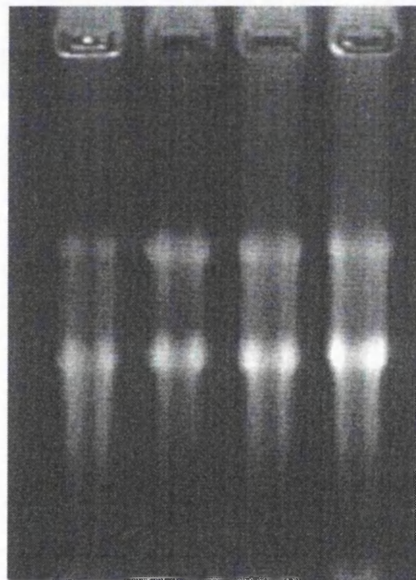
0 8 24 48



β-Actin



Defensin



18S

the inceased mRNA abundance, other factors like mRNA stabilisation effects cannot be excluded as playing a part in NB4 cells. One disparity between the NB4 cells and the HL60-based study, which may be accounted for by such a difference, is that the level of *defensin* mRNA does not return rapidly to uninduced levels after reaching peak abundance. This may however also be related to the different time course of differentiation where the HL60 cells showed peak *defensin* message abundance after 4 (as opposed to 2) days. When compared to the pattern of expression of *defensin* in normal haematopoiesis, the induction of *defensin* expression in the NB4 cells closely follows this time course whilst the down regulation, which is normally seen during terminal stages of granulocytic differentiation, is not replicated in this *in vitro* model. For this reason I decided to concentrate my studies on *defensin* up-regulation as opposed to terminal down-regulation.

A reporter gene driven by the β-*actin* promoter was used in transfection studies (see Chapter12), in NB4 cells, so a repeat Northern was performed to ensure that the β-*actin* mRNA was not increased with differentiation ensuring it to be a good control. Ethidium bromide staining of the total RNA served as a loading control (Fig. 13). Whilst there is little change in the abundance of the β-actin, there is a 10 to 12-fold increase in *defensin* message (as measured by densitometry).

Northern blots of NB4 cells induced to differentiate with vitamin D3 and TPA show a total absence of *defensin* mRNA by the 24 hour time point (Fig. 12). This is in accordance with what has been seen in the case of monocytic differentiation of HL60 myeloblast cells.

Chapter 7 : *In vitro* protein binding to the HNP-1 *defensin* promoter

Initial studies were performed on 240 base pairs of sequence upstream of the main transcriptional start site. These included both footprinting experiments and electrophoretic mobility shift assays (EMSAs). Both others and ourselves identified the main transcriptional start site as being 24 nucleotides downstream from the 3'end of the TATAA box (52;364).

7.1 DNAse 1 footprinting

200 μg of nuclear protein extract from undifferentiated NB4 cells (which expresses *defensin* at a low level), ATRA- induced NB4 cells - 40 hours ATRA exposure (high level *defensin* expression) and TPA- and Vitamin D3- induced NB4 cells - 24 hours inducer exposure (no *defensin* mRNA) were used in the initial experiments. These nuclear proteins are incubated with a DNA duplex fragment representing the above mentioned sequence labelled on only one strand, prior to addition of DNAse1 at known concentrations for a fixed period of time.

7.1.1 Changes in footprints on the defensin promoter with differentiation

Figs. 14 and 15 show such experiments with the positive (sense) and negative (antisense) strands labelled respectively. The protein-DNA complexes are incubated with increasing concentrations (labelled as 1,2,3) of DNAse1 to assess the extent of

Fig. 14 DNAse1 footprinting of the defensin upstream sequence -240/+15 (sense strand).

The labelled DNA was incubated with 200 μg of nuclear protein and DNAse1 at different dilutions. The numeration to the left of the panel indicates the position on the defensin promoter of the 5' extremity of the labelled fragment migrating at that position. This is calculated using a similarly labelled DNA fragment cleaved in G+A specific positions (Maxam-Gilbert chemical cleavage reaction). Lane (B), is a control digestion following incubation with 200μg of bovine serum albumen to exclude non-specific protein binding effects. Lanes 1, 2 and 3 represent 0.5, 1.0 and 1.5 μl of a 20-fold dilution of DNAse1, incubated with the DNA. U, G and M signify, undifferentiated, granulocytic and monocytic NB4 nuclear extract respectively.

Red boxes to the right of the gel panel indicate sequences protected from DNAse1 digestion. The lettering to the right indicates which cellular extracts protect the DNA.

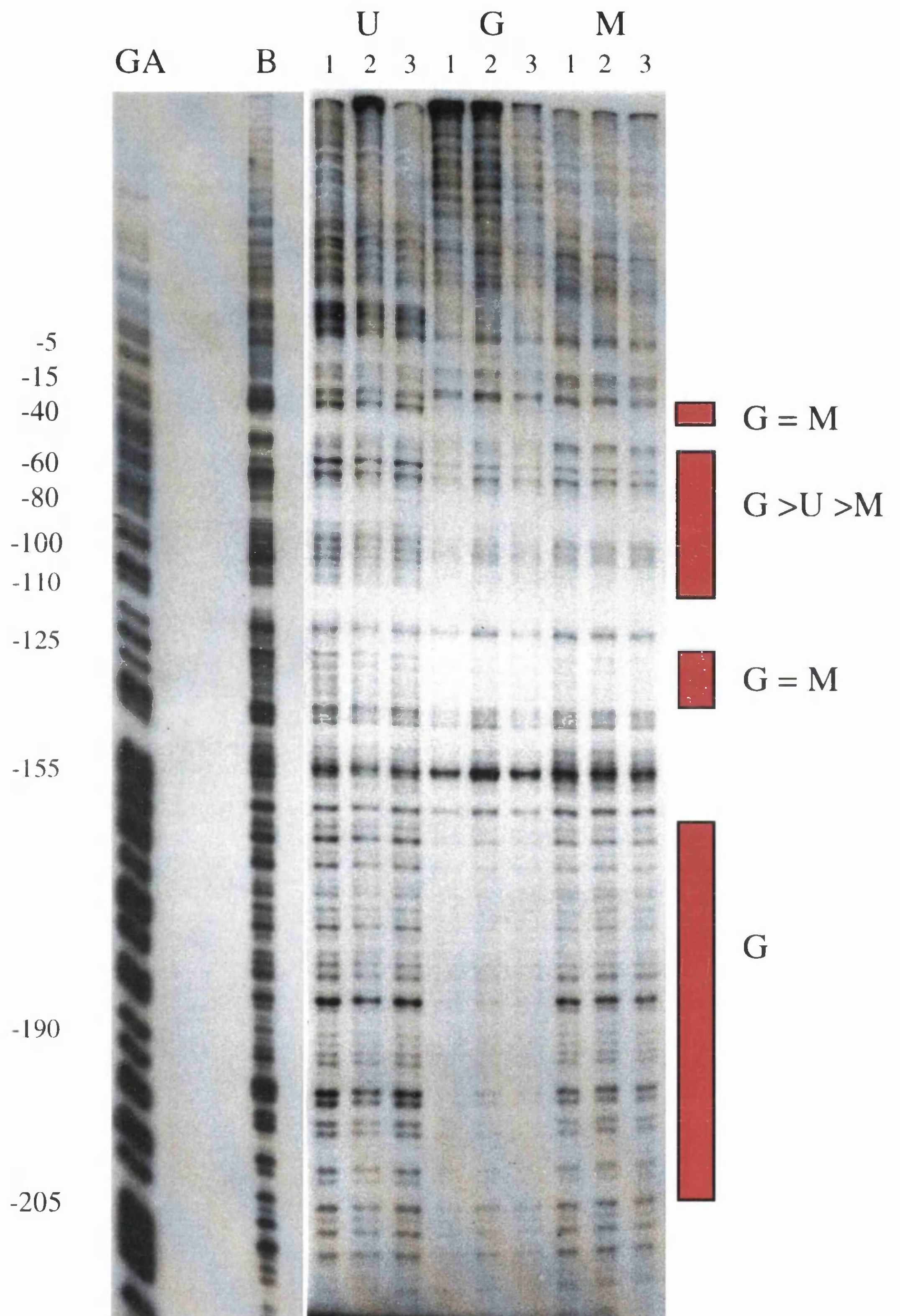
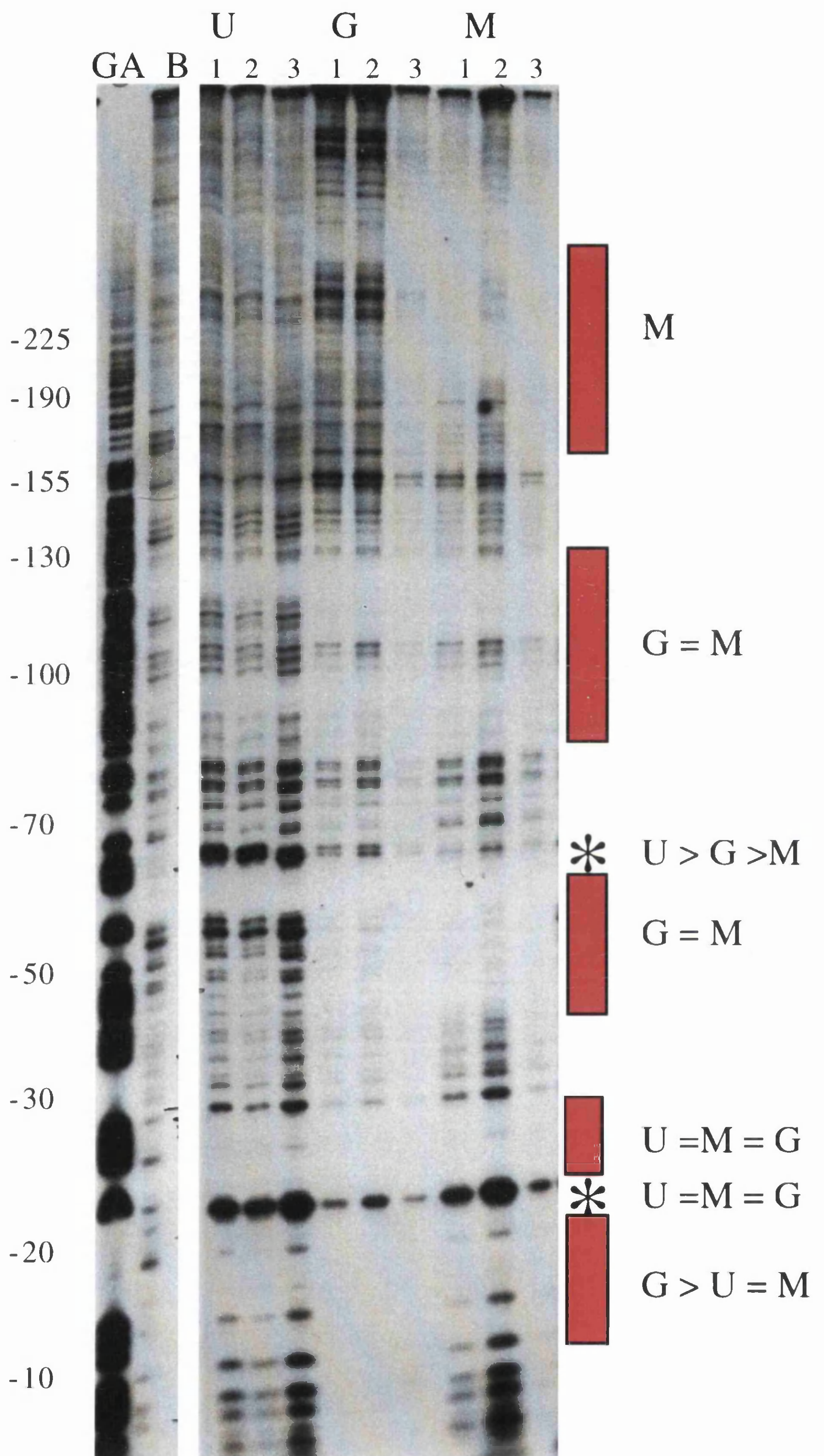

U
G
M
GA
B
1
2
3
1
2
3
1
2
3
-5
-15
-40
-60
-80
-100
-110
-125
-155
-190
-205
G = M
G >U >M
G = M
G

Fig.15 DNAse1 footprinting of the defensin upstream sequence –240/+15 (antisense strand).
The labelled DNA was incubated with 200 μg of nuclear protein and DNAse1 at different dilutions. The numeration to the left of the panel indicates the position on the defensin promoter of the 5' extremity of the labelled fragment migrating at that position. This is calculated using a similarly labelled DNA fragment cleaved in G+A specific positions (Maxam-Gilbert chemical cleavage reaction). Lane (B), is a control digestion following incubation with 200μg of bovine serum albumen to exclude non-specific protein binding effects. Lanes 1, 2 and 3 represent 0.5, 1.0 and 1.5 μl of a 20-fold dilution of DNAse1, incubated with the DNA. U, G and M signify, undifferentiated, granulocytic and monocytic NB4 nuclear extract respectively.
Red boxes to the right of the gel panel indicate sequences protected from DNAse1 digestion. The asterisks indicate the presence of marked hypersensitivity to DNAse1 digestion. The lettering to the right of the gel indicates which cellular extracts protect the DNA or induce hypersensitivity.


U
G
M
GA
B
1
2
3
1
2
3
1
2
3
-225
-190
-155
-130
-100
-70
-50
-30
-20
-10
M
G = M
U > G >M
G = M
U =M = G
U =M = G
G > U = M

protection provided. The control lane (B) is also shown where an equivalent amount (200μg) of bovine serum albumen was added to the same labelled DNA and was incubated with an intermediate concentration (as in lane 2) of DNAse1.

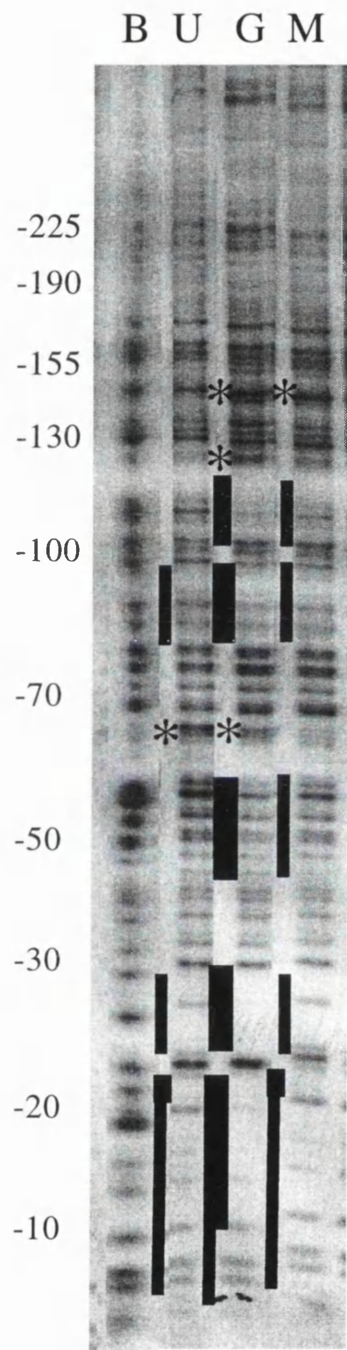
Protected sequences (footprints) are clearly visible (the more obvious of these have been indicated by an adjacent red rectangle), such as the protection of the TATAA box sequence between nucleotides -22 and -30 on the antisense strand. However the large amount of nuclear extract may obscure differences between the different extracts resulting due to sufficient amounts of transcription factors being added in each case resulting in similar footprints.

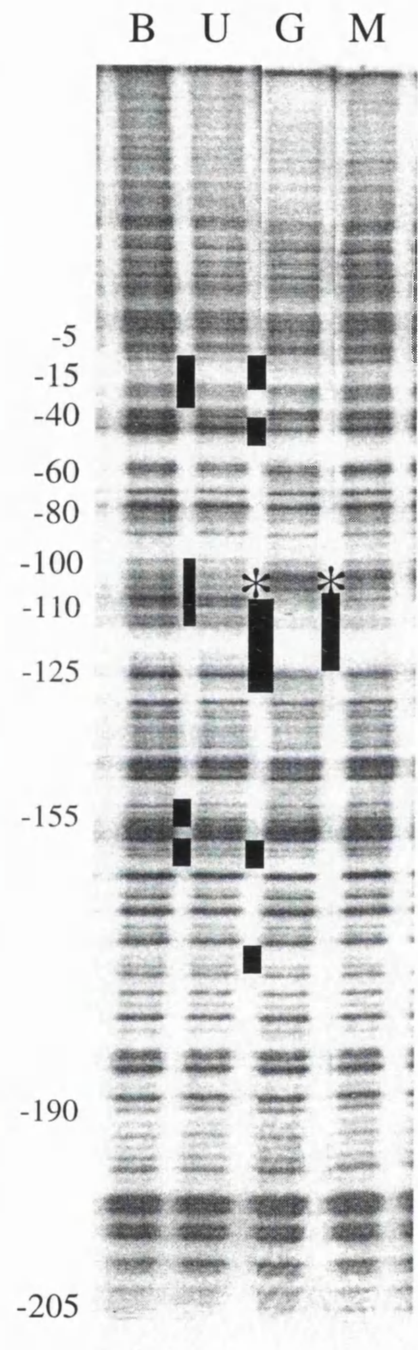
Therefore these experiments were repeated using just 40μg of nuclear extract in order that only sequences bound with highest affinity by the various nuclear extracts would be protected (Fig. 16). The results are diagrammatically represented in Fig. 17 where the footprinted sequences of DNA are indicated by a rectangular block the thickness of which represents the strength of protection seen.

In the presence of undifferentiated NB4 extract, a strongly hypersensitive site at position -63, on the negative strand was the major recognised feature. This hypersite was also detected with granulocytic but not with monocytic nuclear extracts. In addition, granulocytic extract (and monocytic extract to a lesser extent) protected a sequence stretching from -62 to -50, just downstream of the above mentioned hypersite and another stretch of sequence starting from the TATA box at -30 downstream till -10 (Fig. 16). These sequences close to the initiation site were also protected weakly by undifferentiated NB4 nuclear proteins.

Using this reduced amount of nuclear protein (40μg), the TATA box sequence was strongly protected only by granulocytic nuclear extract indicating that high affinity binding to the site (possibly by a component of the basic transcription machinery) only

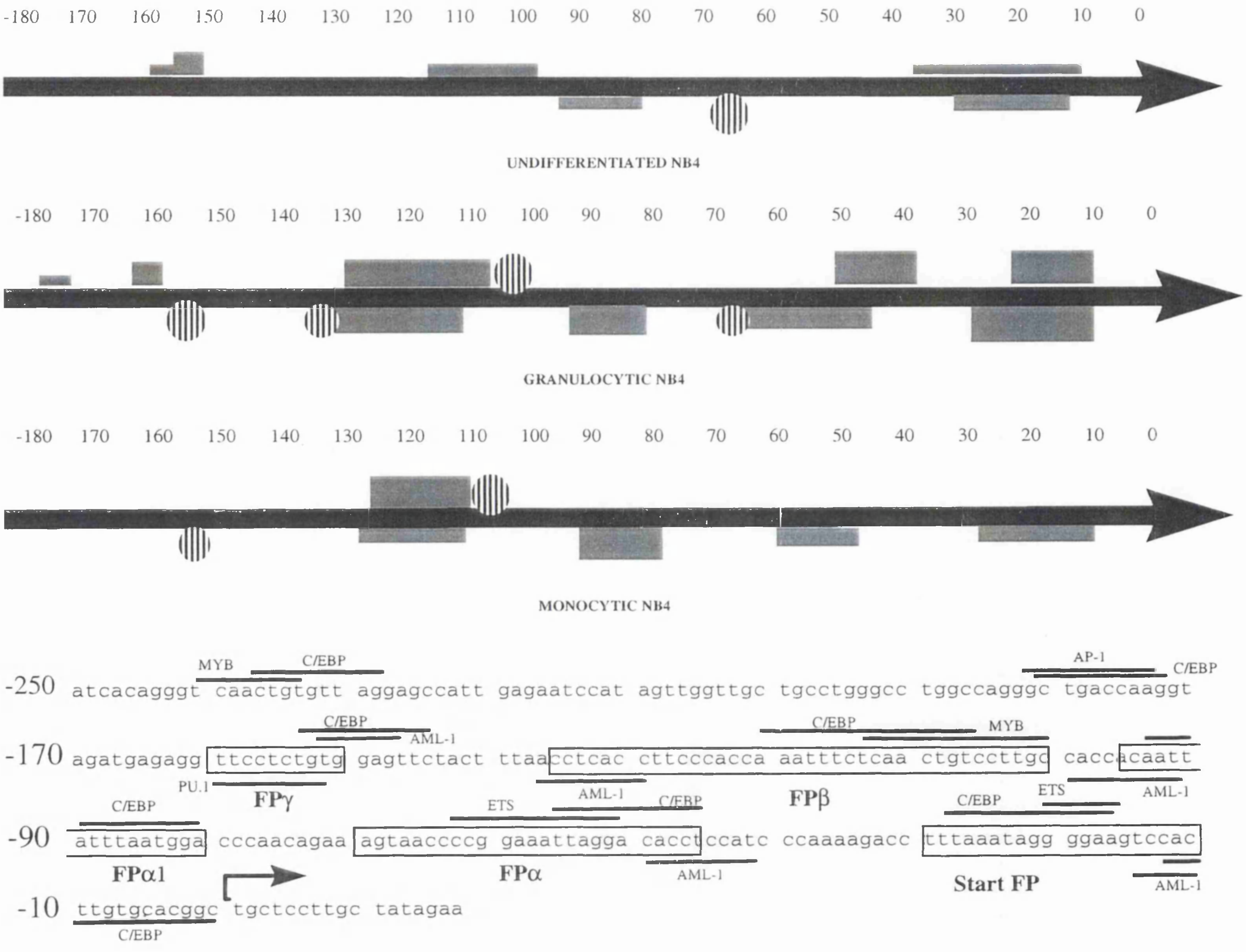
Fig.16 DNAse1 footprinting of the defensin –240/+15 upstream sequence using 40μg of nuclear extract with 0.5U of DNAse1. Numeration to the left of the panel indicates the position of the unlabelled extremity of the fragment as previously. B, U, G and M indicate Bovine serum albumen, Undifferentiated NB4 extract, Granulocytic NB4 extract and Monocytic NB4 extract respectively. Black rectangular boxes next to each lane identify sequences protected from DNAse1 digestion in comparison to the albumen control- the width of these boxes indicates the strength of the protection. Asterisks show areas of increased DNAse1 sensitivity compared to the albumen control.





- Strand   + Strand

Fig.17 Diagrammatic representation of defensin promoter DNAse1 footprinting experiments with 40μg of nuclear protein extract. The long dark arrows represent the defensin upstream sequence and numeration above this indicates the position of particular features. Rectangular boxes indicate protected sequences on the sense (above the arrow) or antisense (below the arrow) strands, thickness signifying increased protection. Hatched ellipses indicates DNAse1 hypersensitive sites with the size of the ellipses indicating the extent of hypersensitivity.
The sequence of the sense strand of the defensin promoter fragment is indicated in the lower part of the figure with the major footprinted sequences indicated in boxes. Also shown by black lines are potential transcription factor binding sites identified by computer assisted searches.



-180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
UNDIFFERENTIATED NB4
-180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
GRANULOCYTIC NB4
-180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
MONOCYTIC NB4
MYB
C/EBP
AP-1
C/EBP
-250 atcacagggt caactgtgtt aggagccatt gagaatccat agttggttgc tgcctgggcc tggccagggc tgaccaaggt
C/EBP
AML-1
C/EBP
MYB
-170 agatgagagg ttcctctgtg gagttctact ttaacctcac cttcccacca aatttctcaa ctgtccttgc caccacaatt
PU.1
FPγ
ETS
AML-1
C/EBP
FPβ
C/EBP
ETS
AML-1
C/EBP
-90 atttaatgga cccaacagaa agtaaccccg gaaattagga cacctccatc ccaaaagacc tttaaatagg ggaagtccac
FPα1
FPα
AML-1
Start FP
AML-1
-10 ttgtgcacggc tgctccttgc tatagaa
C/EBP

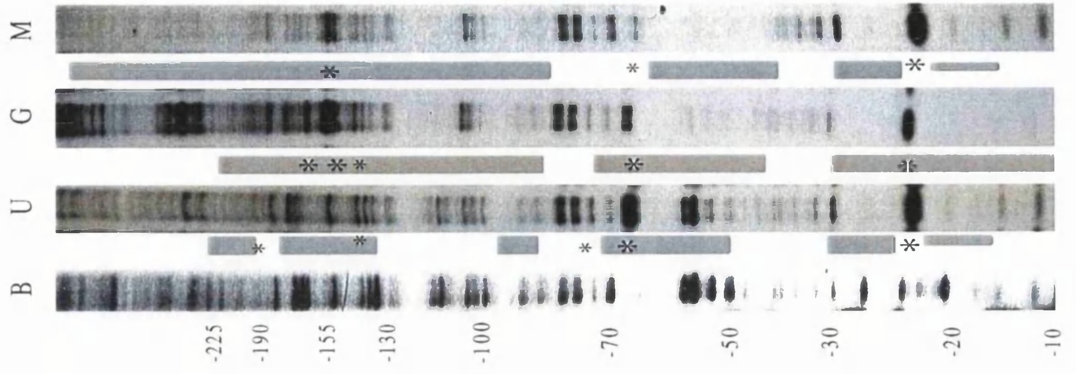
occurs during granulocyte differentiation. This correlates with high level *defensin* gene expression only being seen in granulocytic NB4 cells.

Extracts from both granulocytic and monocytic differentiated NB4 nuclei protected sequences -125/-110 on both strands with associated hypersites being detected just downstream of this sequence on the positive strand and just upstream on the negative strand. The sequence between position -80/-90 was also protected to some extent by all three extracts on the negative strand. Hypersensitive sites were seen at position -155 on the negative strand in the presence of both granulocytic and monocytic nuclear extracts, though this was much more clearly seen with greater amounts (200μg) of nuclear protein (Fig.18) where it is detectable on both strands. Some weak protection was also seen around this site on the positive strand with undifferentiated nuclear extract.

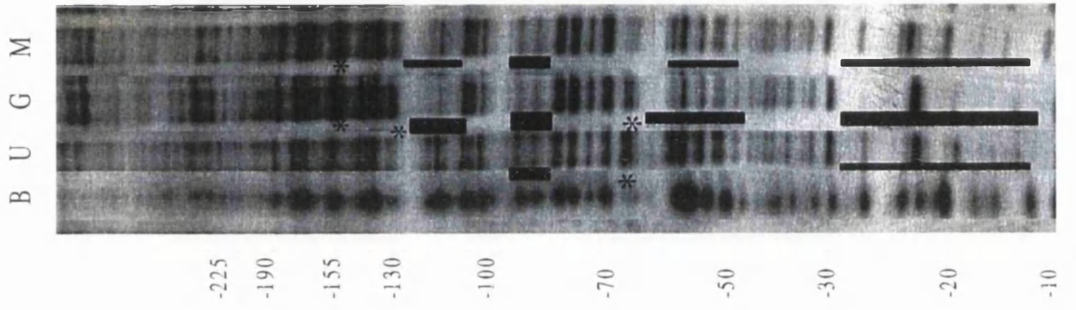
Various other sequences are protected when using 200μg of nuclear protein but are less likely to be functionally significant *in vivo*, being less specifically bound.
They include one sequence specifically protected by granulocytic proteins, which extends from position -210 till -160 on the sense strand. Another long footprint was seen with monocytic protein, from sequence -240 to -200 on the anyisense strand.

A comparison between the footprints obtained with 40 and with 200μg of nuclear protein extract is seen in Fig. 18 and the differences between the two experiments are represented diagrammatically in Fig. 19. The results from the 200μg experiment are represented by grey rectangles the darkness of which is an indication of the strength of protection. The results from the 40μg experiments are indicated by the presence of unfilled or colour filled boxes with the thickness of each box indicating the strength of protection. Hypersensitive sites are indicated by hatched circles, which are also seen in the 40μg experiment if they lie on a white circle.

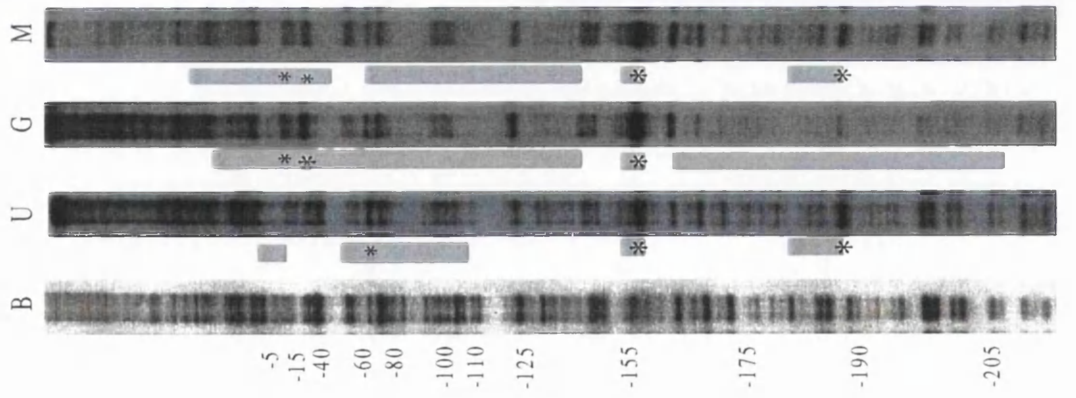
Fig. 18 Comparison of the DNAse1 protection (footprinting) seen with different amounts of nuclear proteins. Numeration to the left of the panel indicates the position of the unlabelled extremity of the fragment as previously. B, U, G and M indicate Bovine serum albumen, Undifferentiated NB4 extract, Granulocytic NB4 extract and Monocytic NB4 extract respectively. Black boxes and grey boxes to the left of each lane indicate the footprinted sequences.



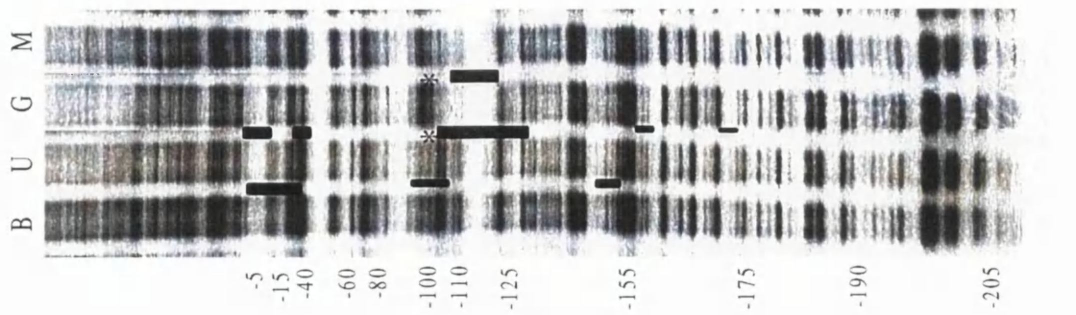
-Strand(200μg protein)



-Strand(40μg protein)

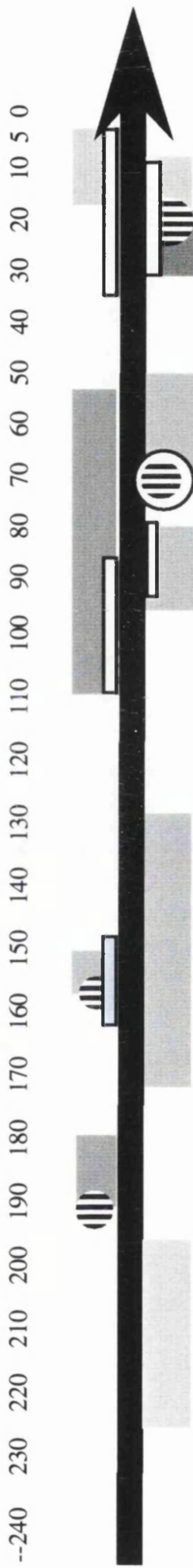


+Strand (200μg protein)

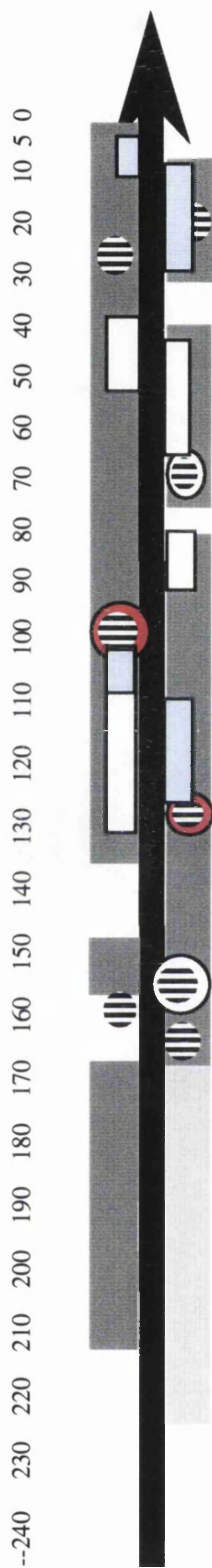


+Strand(40μg protein)

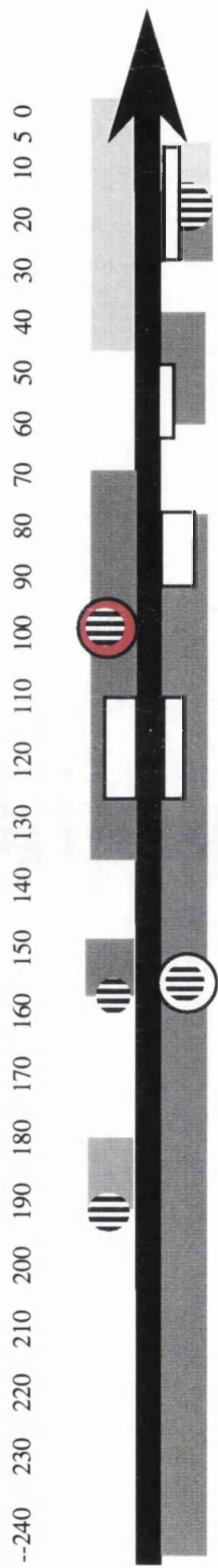
Fig.19 Diagrammatic representation of the differences in footprinting studies with different amounts of nuclear protein extract. Arrows represent the promoter as in Figure 17. Grey rectangles represent the footprints seen with 200μg of nuclear extract; the darkness of each rectangle indicates the strength of the protection. White rectangles indicate the footprints seen with 40μg of nuclear protein extract and the thickness of the rectangle signifies the extent of protection seen. Hatched ellipses indicate hypersensitive sites seen with 200μg of protein and those lying within a white ellipses are also seen when using 40μg. Lilac rectangles indicate sequences protected to a different degree by different extracts when 40μg of protein was used but to a similar extent when 200μg of extract was used. Red ellipses indicate hypersensitive sites seen when using 40μg of nuclear protein which were not seen with 200μg of nuclear extract.



UNDIFFERENTIATED NB4



GRANULOCYTIC NB4



MONOCYTIC NB4

7.1.2 Preliminary interpretation of Footprinting Results

The sequences protected to some extent on either or both strands by 40μg of nuclear protein extract (and therefore the most likely to be functionally relevant) were grouped into continuous overlapping composites independent of the strand protected and named accordingly. This nomenclature will be used in referring to these sequences during further studies resulting from these initial experiments. These included three major footprints: -

-30/-10 Start Site Footprint (which includes the TATA sequence)

-70/-45 FP α

-135/-100 FP β

And two much smaller footprinted sequences whose names refer to their position in relation to the previously mentioned named footprints.

-95/-80 FPα1

-160/-150 FPγ

Using the computer-based search engines MatInspector, TFSearch (373) and GCG Wisconsin Package Version 9 (Genetics Computer Group, Madison, Wis.), I looked for potential transcription factor binding sites with the sequence of the 240 bases upstream of the *defensin* start site. Numerous potential binding sites were found many of which overlapped to some extent with the footprinted sequences. These transcription factor binding sites are marked on Fig. 17 (bottom half) together with a schematic representation of the footprinted sequences (boxed in on the promoter) so as to show the overlap.

The main aim of the footprint studies was to show differences in binding with differentiation and between lineages and to correlate this with the activity of the *defensin* promoter in these situations. Clear differences were indeed seen. Differentiated nuclear protein from either lineage resulted in markedly increased binding to footprints α, α1 and β. The sequences which were increasingly protected by differentiated protein extract overlap with the binding sites for C/EBP- and AML-family transcription factors. The appearance of the hypersite at position -155 also correlates with differentiation and overlies a potential binding site for the Ets transcription factor PU.1. The increased protection at the binding sites of all these factors by differentiated nuclear extract correlates well with their known expression profiles. They are up-regulated during granulocytic differentiation both in myeloid cell lines and in primary myeloid cells (21;338;374).

The feature that seems to most characterise the undifferentiated state is the strong hypersite found at the most upstream part of FPα. Whilst this hypersite is considerably less obvious with granulocytic extract, it is still present, yet it is totally absent with monocytic extract. It lies just upstream of a strong potential binding site for another Ets transcription factor. Ets factors (such as PU.1) are known to be capable of bending DNA and as such may be responsible for the hypersites found both at this position and at position –155 (375).The other hypersites found at the upstream and downstream limits of FPβ may be caused by AML-1 and Myb respectively, both of which have been shown to bend DNA (376;377).

By correlating the footprints with the level of *defensin* mRNA expressed in each case, it appears that the presence of the FPα hypersite (probably a result of Ets factor binding) is required for the gene to be at all active. However it is the presence of this hypersite together with protection of footprints β, α1 and α downstream of the

hypersite, that correlates with high-level gene expression in granulocytic NB4 cells. The presence of the factors protecting these latter sequences in differentiated cells (possibly C/EBP and/or AML family) without the associated presence of the factor causing the FPα hypersite were not associated with even minimal gene expression.

Correlations between changes in promoter binding and the levels of *defensin* mRNA detected are based on the assumption that transcriptional regulation is mainly responsible for regulation of these mRNA levels. Studies of *defensin* gene expression in myeloid cell lines have indicated this to be the likely case (52).

One of the more interesting points recognised during these footprinting studies is that the footprinting seen when using a lesser amount of nuclear protein (40μg) was not uniformly weaker than that seen with a greater amount of nuclear protein (200μg). Nor were the differences between different types of extracts most clearly shown with greater amount of extract. In Fig. 19, the lilac-filled boxes indicate footprints which are quite clearly more specifically protected by one particular extract when using 40μg but which were equally protected by different extracts when 200μg of protein were used.

This is not so remarkable in itself though important in designing experiments. It suggests that the threshold amount of a factor X, required to bind to and protect a particular segment of DNA, is found in differing amounts of total nuclear protein from different cells. This depends on the abundance of that particular factor within the nuclear extract at each stage of differentiation. More surprising are the hypersensitive sites (seen as hatched circles upon a red background in Fig.19) which are detected when using 40 but not with 200μg of nuclear extract. The explanation for this is unclear but one possibility may be that when using 200μg of nuclear protein, unbound excess of one nuclear factor may interact with other factors and prevent their binding to DNA.

In summary, footprinting studies suggest the importance of factor binding to the upstream of FPα for minimal gene activity. Factor binding to other sequences (FPs α, α1, β) upon differentiation, together with the FPα hypersite correlate with high level activity but similar sequence protection in the absence of the FPα hypersite does not.

7.2 Electrophoretic Mobility Shift Sssay on the defensin promoter

7.2.1 Different protein binding with differentiation

The whole -240/+15 upstream sequence of the *defensin* gene was labelled and used as a probe in electrophoretic mobility shift assays using extracts from NB4 cells in different stages/lineages of differentiation and also using extract from an HL60 sub-line which had lost *defensin* expression. The results of this experiment are shown in Fig. 20. Undifferentiated or granulocytic NB4 nuclear extracts (from cells in which *defensin* is expressed) retard the progress of the labelled probe through the 4% polyacrylamide gel considerably more than do the extracts of monocytic NB4 cells or HL60 cells both of which are not expressing *defensin.* This would suggest that either a lot more proteins bind directly to the probe sequence in the case of the former two extracts, or that larger protein complexes (thereby resulting in more retardation) are involved as a result of protein-protein interaction. The footprinting results suggest the latter hypothesis to be the case because monocytic extract provides more protection (and therefore includes more direct DNA-binding proteins than undifferentiated extract). Undifferentiated and granulocytic NB4 cells are the two cell types which express *defensin.* It is therefore tempting to speculate that this increased retardation is a result of the basal transcription

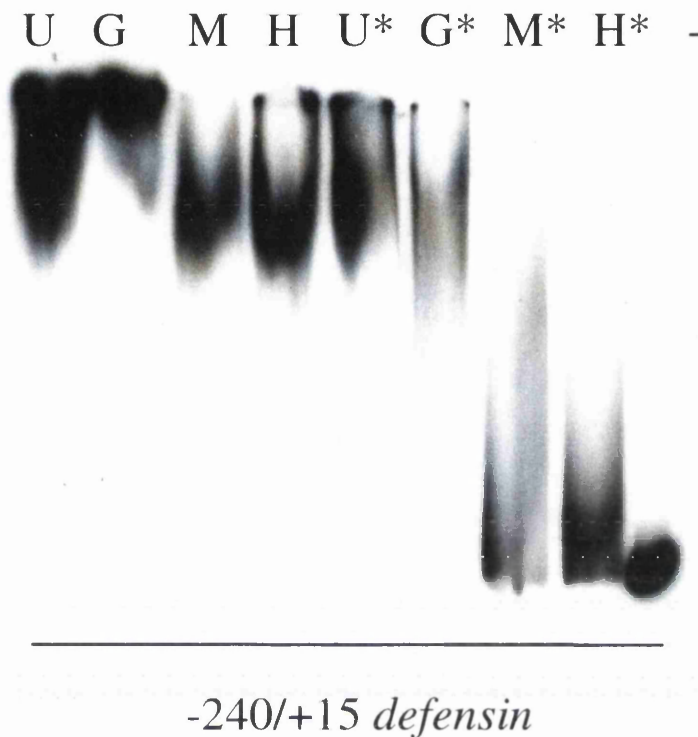


Fig. 20 Electrophoretic mobility shift assay (EMSA) of the defensin upstream sequence –240/+15 with different nuclear protein extracts. 5μg of nuclear protein from Undifferentiated NB4 cells (U), Granulocytic NB4 cells (G), Monocytic NB4 cells (M), or HL60 cells (H) were incubated with labelled probe in the presence of buffer 2. The reactions were then loaded and run into a 5% polyacrylamide gel. Other incubations were also performed with the same nuclear protein extracts and probe and a 50-fold molar excess of unlabelled probe sequence (signified by an "*"). The last lane, labelled (-) is a similar reaction without the nuclear extract.

machinery (or components thereof) binding onto the DNA in combination with transcription factors found in these cells but lacking in the others.

Competition experiments with 50-fold molar excess of unlabelled DNA probe show a differing pattern of band shifts. In the presence of monocytic NB4 or HL60 nuclear extract, the retarded probe-protein complex is completely lost. However, a band is still seen with undifferentiated or granulocytic nuclear extract, though of an increased mobility, suggesting that some but not all of the bound components were removed by competition. Such a picture upon self competition, usually indicates the presence of non-specific protein binding. Another possibility is that a one (or more) transcription factor/s present in granulocytic and undifferentiated NB4 extracts (possibly those responsible for the -63 hypersite) are not completely competed away by a 50 fold molar excess of unlabelled DNA. These factors alone may be able to tether part of the basal transcription machinery to the labelled DNA resulting in a still markedly retarded band. As can be seen below, greater excess of unlabelled oligonucleotide did indeed completely compete away granulocytic NB4 protein binding.

7.2.2 Individual footprint sequences cannot compete promoter-bound complex

An attempt was made to isolate which sites, were required for the protein binding seen with the extract of expressing cells. The labelled -240/+15 defensin upstream sequence was competed with 200-fold molar unlabelled excess of the same long sequence in the presence of granulocytic NB4 nuclear extract. Competition was also performed using 200-fold excess of unlabelled oligonucleotides representing the different footprinted sequences as well as recognised binding sites for the transcription factors suspected to bind the *defensin* promoter. None of the individual

oligonucleotides where capable of disrupting the retarded band as much as the complete sequence did (Fig.21). This phenomenon has been recognised (158) and is thought to be due to single factor DNA-binding being incapable of competing with the stability of a multifactorial complex bound to DNA including the said factor. A certain increase in mobility was however seen with most of the oligonuceotides and there was also some reduction in the intensity of binding. The significance of these changes are hard to interpret in the context of the whole 255 base pairs, however. Therefore n order to reduce the complexity of protein complexes formed in these EMSAs and be able to perform more specific experiments we progressed to studying binding to the main footprinted sequences in isolation.

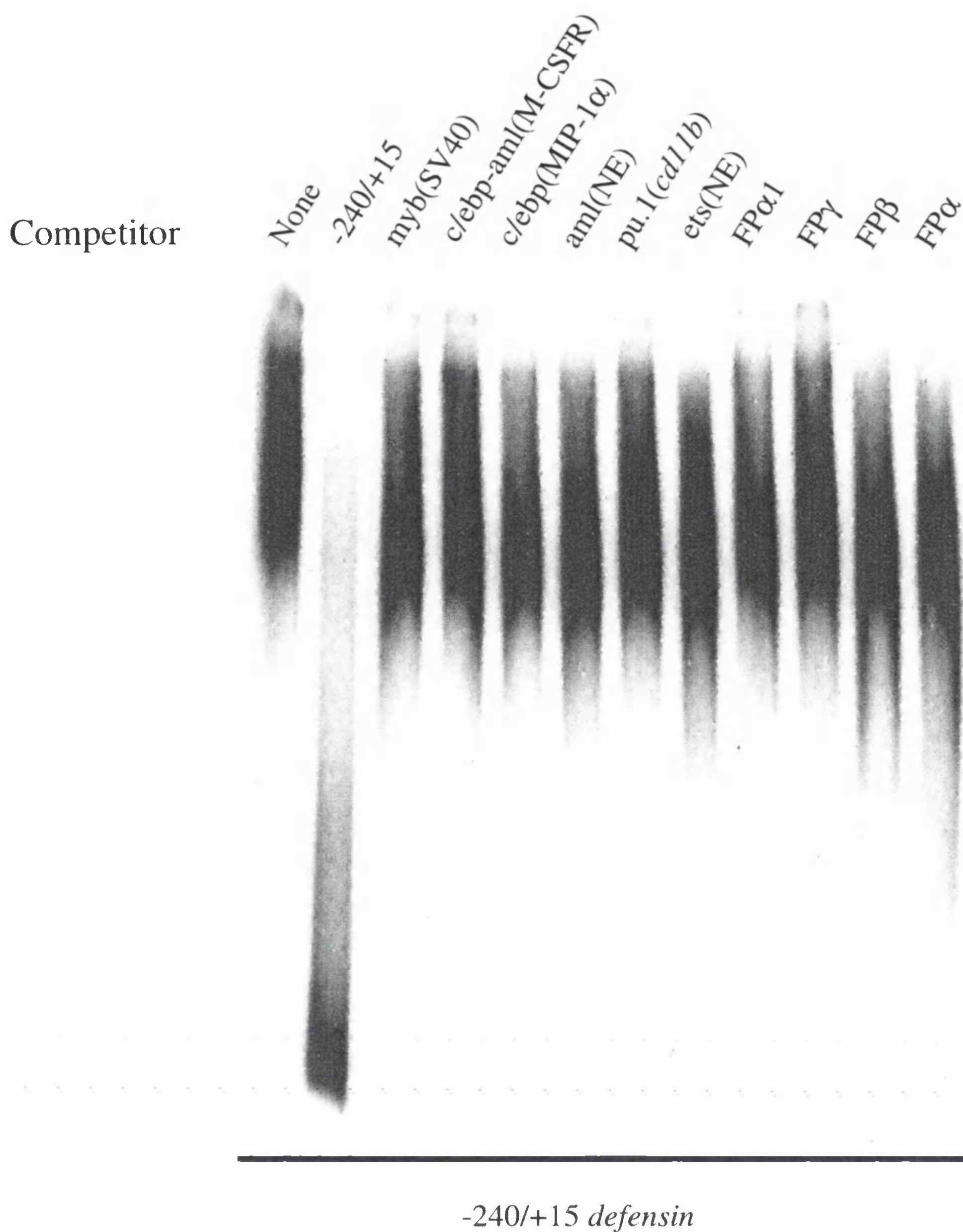


Fig.21 Competition EMSA with –240/+15 promoter fragment as the [γ-^{32}P]-labelled probe sequence. 5μg of granulocytic NB4 nuclear protein extract was incubated together with the probe and a 200-fold molar excess of the indicated competitor sequence in the presence of buffer 2. Incubated reactions are then loaded and run on a 4% polyacrylamide gel. The sequences of the different competitor oligonucleotides are documented in the materials and methods section.

Chapter 8 : Analysis of Footprint-bound proteins by EMSA

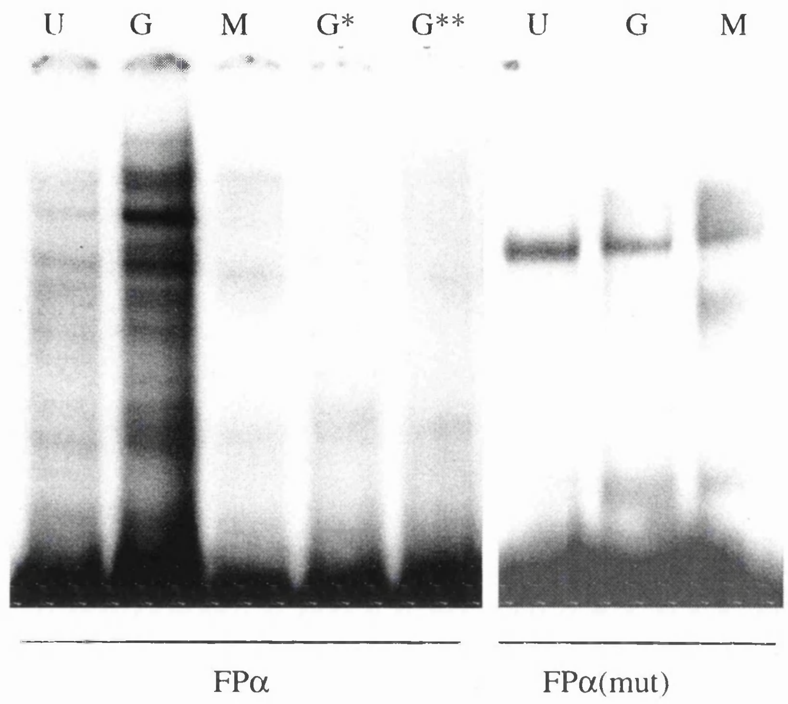
8.1 Analysis of FPα-binding proteins

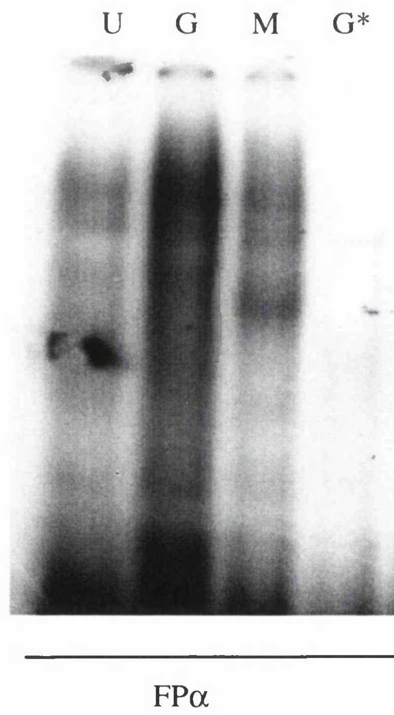
8.1.1 Changes in FPα binding with differentiation

Using a oligonucleotide incorporating the FPα binding site (-76/-39), EMSAs were performed using extract from undifferentiated, granulocytic and monocytic nuclear extract with two different buffers (Fig. 22 a, b). A clear increase in binding is seen with the granulocytic as opposed to the undifferentiated nuclear extract. Monocytic extract also shows less binding than granulocytic but with a different pattern from that seen with the undifferentiated extract. These results all correlate with those obtained by DNAse1 footprinting.

Binding is specific, as can be seen by self competition experiments whilst a recognised Ets-1 binding site also competes away most granulocytic-bound protein suggesting the importance of the ets core binding site (GGAA) within FPα. Using an oligonucleotide identical to the FPα one but mutated in this core ets site (Fig. 22a) as the labelled probe, results in much reduced binding showing that Ets factors are probably bound to this site and may possibly be responsible for co-operative binding of other factors. These results correlate well with recently published data indicating that an Ets factor is bound to this site of the *defensin* promoter in HL60 nuclear extract in a phosphorylation-dependent manner (54).

Fig.22 Changes in nuclear protein binding to FPα with differentiation. (a) 5μg of nuclear protein from undifferentiated (U), granulocytic (G), or monocytic (M) NB4 cells was incubated in buffer 2 with [γ-^{32}P] labelled FPα oligonucleotide. Specificity of binding was confirmed by competition of granulocytic protein binding to the probe with a 200-fold molar excess of unlabelled FPα oligonucleotide (G*) and a 200-fold molar excess of ets site from the murine *neutrophil elastase* promoter (G**). Also shown are similar reactions (also in buffer 2) where the labelled probe sequence is FPα mutated at the core ets site GGAA nucleotides, marked as FPα(mut). All reactions are run on a 6% polyacrylamide gel. (b) Similar EMSA to that shown in Fig.22(a) but with reactions performed using buffer 1. Protein complexes bound within this buffer are also specific as can be seen by competition of granulocytic extract proteins bound to FPα, by a 200-fold molar excess of unlabelled FPα (G*).


U
G
M
G*
G**
U
G
M
a
FPα
FPα(mut)


U
G
M
G*
b
FPα

8.1.2 Characterisation of FPα binding by competition EMSAs

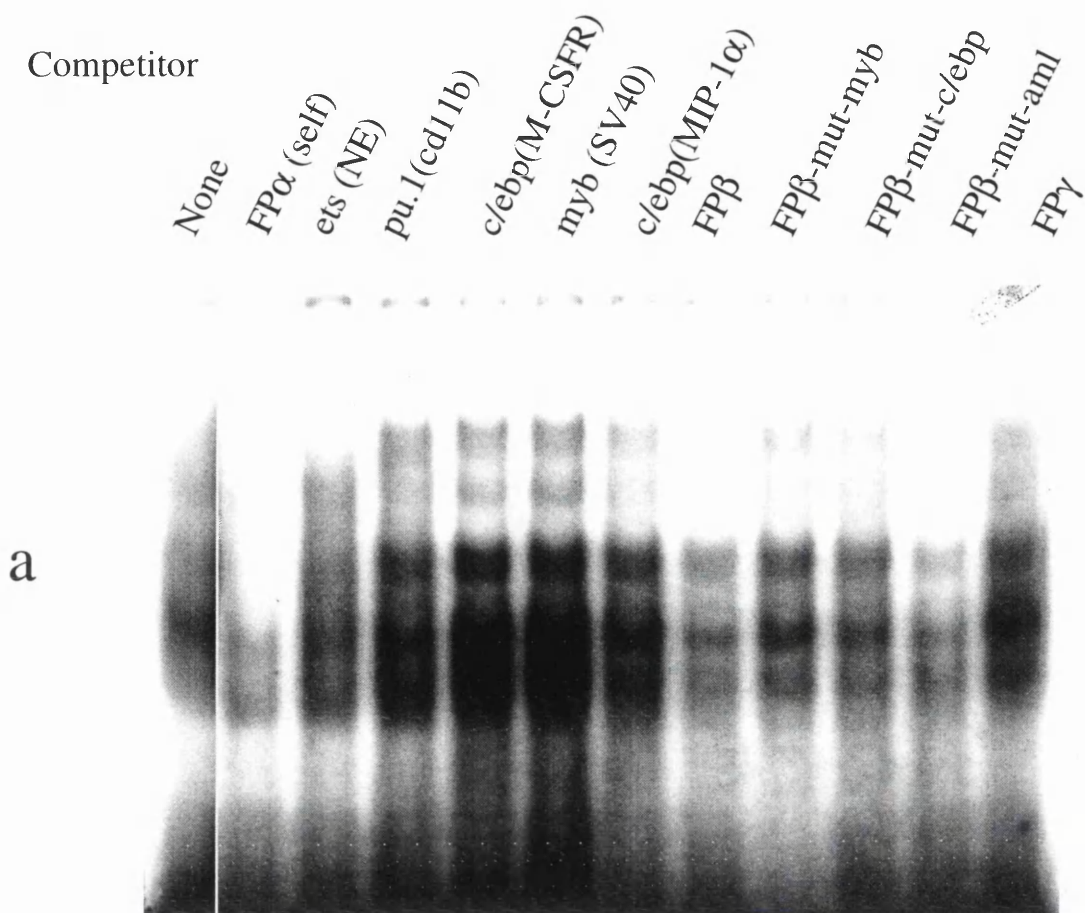
To try and further identify which factors may be bound to the FPα oligonucleotide, competition experiments with different unlabelled oligonucleotides, in excess, were performed (Fig. 23) in the presence of undifferentiated (23a) or granulocytic (23b) nuclear extracts.

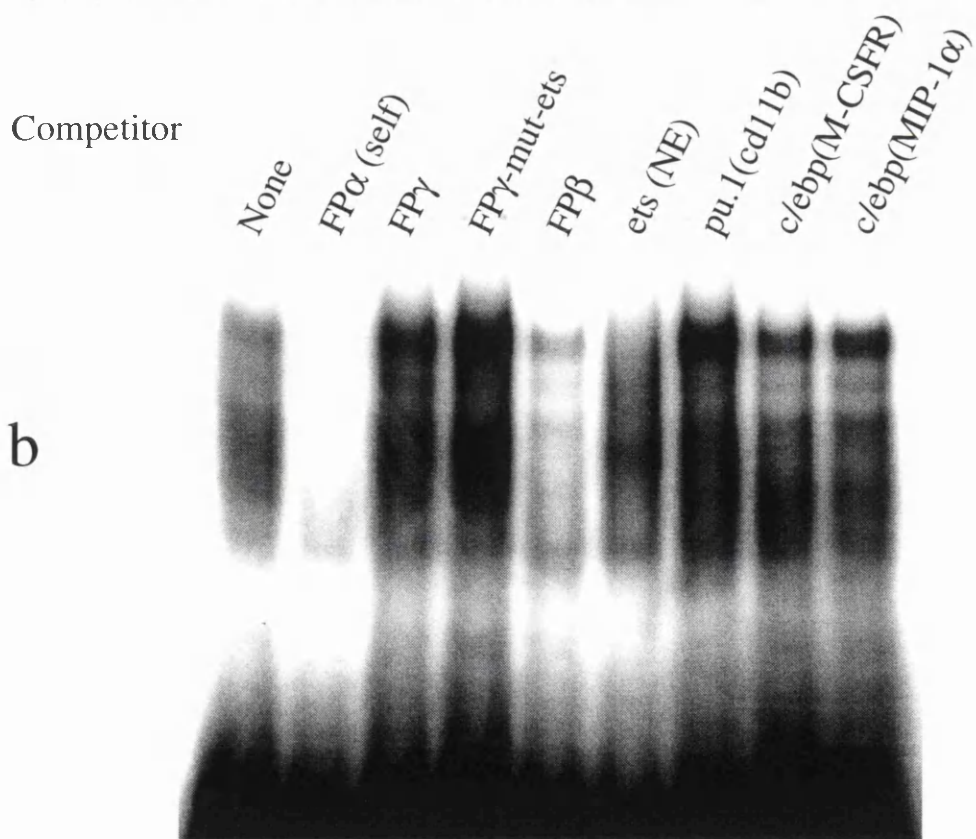
FPβ was able to compete away some complexes formed by undifferentiated extract but this same FPβ sequence mutated at either its potential c/ebp or myb sites was incapable of doing so. This suggests that some factors bound to FPα (possibly C/EBP) are also bound to FPβ. The requirement for the Myb site to be intact for efficient competition suggests a co-operative binding of sorts though co-operative binding between Myb and C/EBP factors has never yet been recognised. Many of the competitor oligonucleotides enhance the formation of certain complexes bound to FPα (seen as bands of high and intermediate mobility).

When using granulocytic differentiated NB4 extract (Fig.23 b), unlabelled FPβ competed binding to some extent, as was the case with undifferentiated extract. A known GABP/PU.1 (Ets) binding site also reduced the formation of certain bands. On the other hand, recognised or potential PU.1 binding sites (including FPγ) as well as recognised C/EBP-binding sites resulted in increased complex formation on the labelled FPα DNA and enhancement in the intensity of a low mobility band. The significance of these findings is discussed later.

AML family proteins are also bound to FPα as can be seen in Fig. 24. A competing oligonucleotide which is a recognised AML-1-binding site (from the mouse neutrophil elastase promoter) inhibits the formation of two complexes whilst a similar oligonucleotide mutated at this site does not also compete them away. An

Fig.23 Competition EMSA of FPα–bound proteins. (a) Analysis of FPα-bound proteins in undifferentiated nuclear extract. 5μg of nuclear extract was incubated in buffer 1 together with 200-fold molar excess of unlabelled competitor as indicated for each reaction and with labelled FPα probe as described in materials and methods. Complexes were separated by electrophoresis through 6% polyacrylamide gel. (b) Competition EMSAs are performed with 200-fold molar excess of the competitor sequence indicated and 5μg of granulocytically differentiated NB4 nuclear protein extract and a [γ-^{32}P] labelled FPα probe sequence in the presence of buffer 1.Complexes are separated as in Fig 23a.


Competitor
None
FPα (self)
ets (NE)
pu.1(cd11b)
c/ebp(M-CSFR)
myb (SV40)
c/ebp(MIP-1α)
FPβ
FPβ-mut-myb
FPβ-mut-c/ebp
FPβ-mut-aml
FPγ
a


Competitor
None
FPα (self)
FPγ
FPγ-mut-ets
FPβ
ets (NE)
pu.1(cd11b)
c/ebp(M-CSFR)
c/ebp(MIP-1α)
b

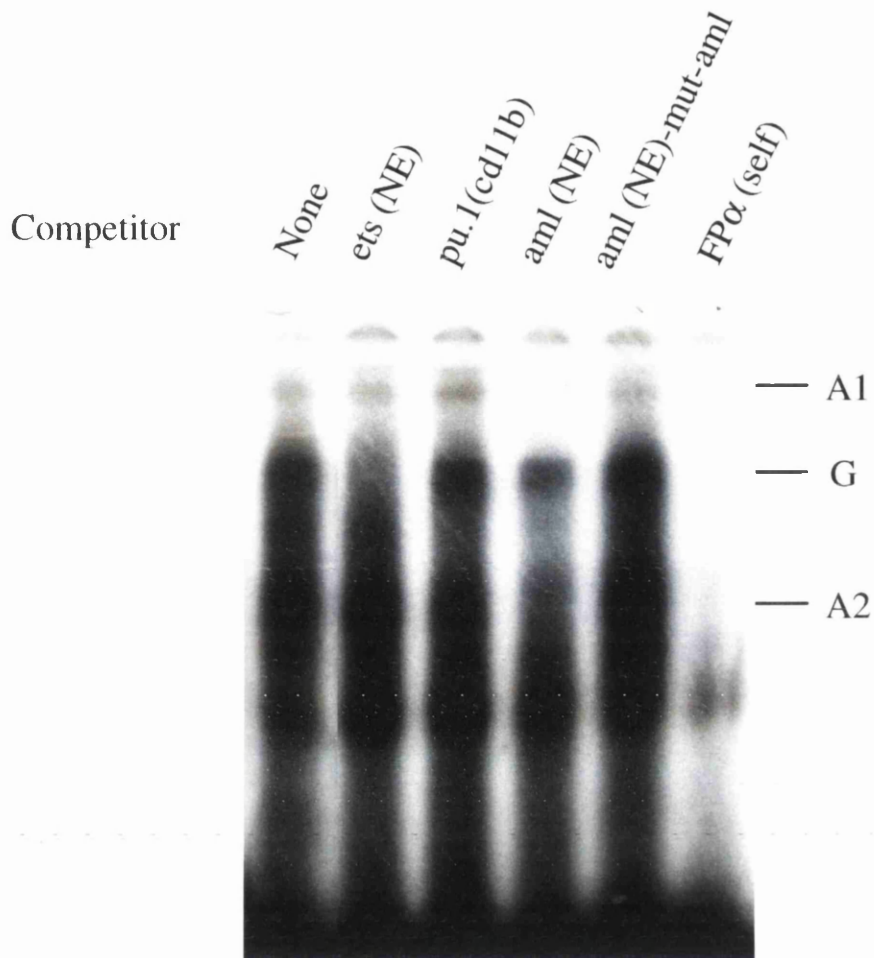


Fig.24 Competition EMSA to assess AML and Ets binding to FPα. 5µg of granulocytic nuclear protein extract was incubated together with a 200-fold molar excess of unlabelled competitor oligonucleotide as indicated together with EMSA buffer 3 and with labelled FPα probe. Complexes were separated through a 6% polyacrylamide gel. A1 and A2 identify two complexes competed by a known AML-1-binding oligonucleotide but not competed by the same oligonucleotide mutated so as not to bind AML-1. G identifies a complex competed by the *neutrophil elastase* (NE) but not the *cd11b* ets site, therefore likely to be GABP

oligonucleotide competitor which is a known binding site for the Ets factor GABP (from the *neutrophil elastase* promoter) competes away the binding of a separate complex (marked G) which was not however successfully competed away by another Ets factor (PU.1) binding site (lane 3).

8.1.4 GABP binds to FPα

These previous studies helped identify the core ets GGAA site within FPα as essential for binding. Closer analysis of the site suggests that there is close resemblance with the consensus binding sequence for the GA-binding protein (GABP). Other studies within our group (T. Jamieson unpublished) showed that binding to this site was sensitive to methylation or even hemimethylation of the site, a recognised feature of GABP binding (378).

Using a pair of antibodies raised against GABP expressed in and purified from *Escherichia coli* (*E.coli*), we attempted to identify whether this factor was indeed binding to FPα both in undifferentiated and granulocytic NB4 nuclear extracts. With undifferentiated extract, two band doublets (marked as 1 and 2 in Fig. 25a) which are not effected by pre-immune serum, are supershifted in the presence of GABPα antibody but not in the presence of GABPβ antibody. Whilst the higher mobility (and probably smaller) of these proteins/complexes (band2) may represent GABPα binding to the DNA alone, the lower mobility complex (band 1) should represent bigger complexes resulting from GABPα binding to the DNA with (or adjacent to) another factor/s.

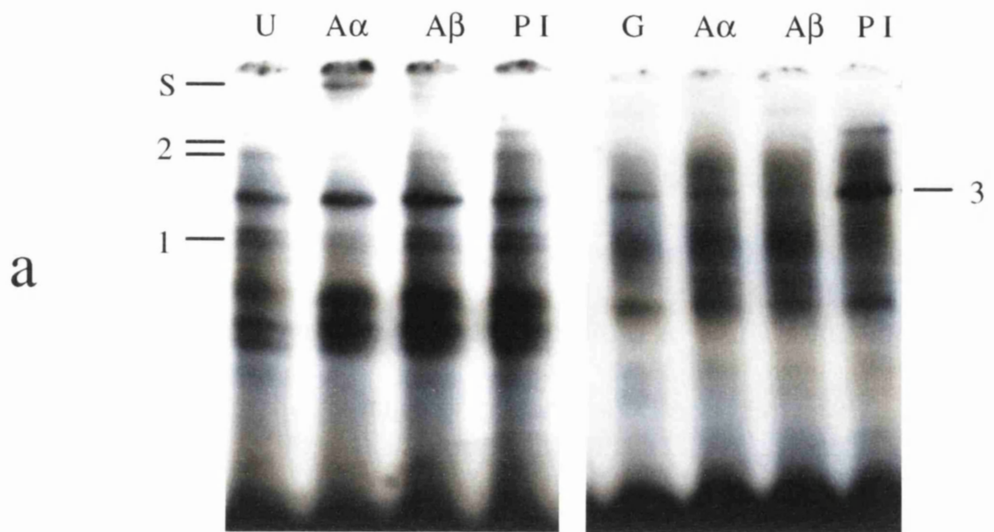
With differentiated extract, such supershifted bands are barely seen at all with GABPα antibody but a reduction in the intensity of another band, (marked as 3) is seen

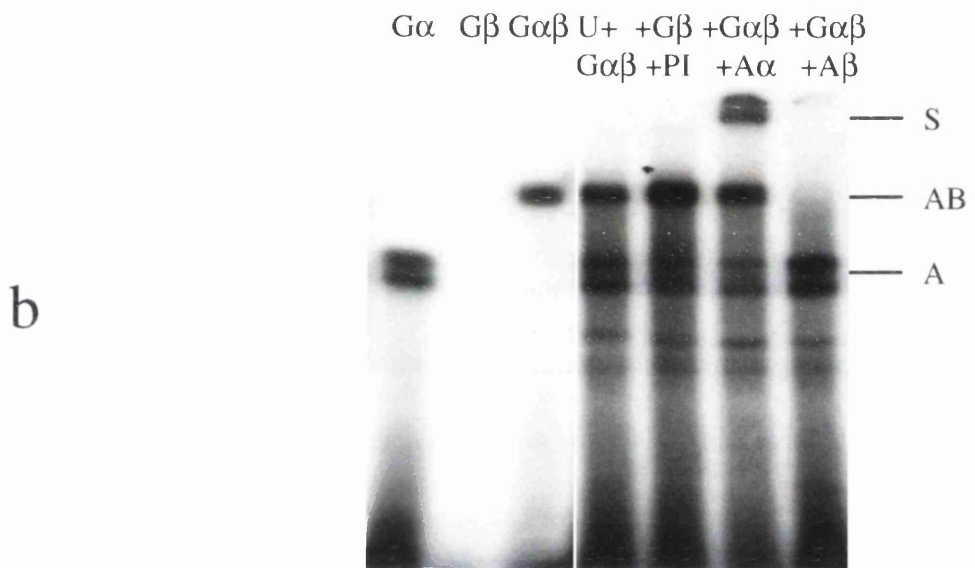
Fig.25 GABP binding to FPα.

(a), 5μg of nuclear protein from NB4 cells - undifferentiated (U), or granulocytic (G) - was incubated in buffer 3 with GABPα antibody (Aα), GABPβ antibody (Aβ) or pre-immune serum (PI). [γ-^{32}P] labelled FPα oligonucleotide was then added. Bound complexes were separated by electrophoresis in a 6% polyacrylamide gel with 0.5xTBE as running buffer. Complex doublets 1 and 2 are supershifted by GABPα antibody resulting in a complex marked as S. Complex 3 (formed with differentiated extract) is disrupted by GABPβ antibody.

(b) 25ng of purified GABPα (Gα) or GABPβ (Gβ) or both (G$\alpha\beta$), in the presence or absence of nuclear protein (10μg) from undifferentiated NB4 cells (U+G$\alpha\beta$), and antibody to GABPα(+Aα), GABPβ(+Aβ) or pre-immune serum (+PI) were incubated in buffer. [γ-^{32}p]-labelled FPα oligonucleotide was added and incubation continued. 0.25xTBE instead of 0.5xTBE was used as electrophoresis running buffer since purified GABP proteins are prevented from binding well under higher salt concentrations. The doublet A represents GABPα alone bound to DNA whilst the band AB represents the bound GABP$\alpha\beta$ heteromer. S indicates a supershifted band on addition of GABPα antibody. Binding of the GABP$\alpha\beta$ complex is prevented by GABPβ antibody with a concomitant increase in GABPα binding.

(c) 25ng of purified GABPα and GABPβ are incubated together with buffer 3 with [γ-^{32}p]-labelled probes FPα, FPα, mutated at the core GGAA-(FPα-mut-ets) and the ets site from the *neutrophil elastase* promoter- ets (NE). As in Fig.25b, 0.25x TBE is used as a running buffer for electrophoresis. Binding of the GABP dimer to FPα is strong and clearly dependent on the FPα core ets site.


U
Aα
Aβ
P I
G
Aα
Aβ
P I
S
2
1
3
a


Gα
Gβ
Gαβ
U+ Gαβ
+Gβ +PI
+Gαβ +Aα
+Gαβ +Aβ
S
AB
A
b

FPα
FPα-mut-ets
ets (NE)
c

with the GABPβ antibody. In order to confirm the effects of the two antibodies, and the binding characteristics of the components of the GABP complex, similar EMSAs were performed using purified GABP proteins made in *E.coli* and labelled FPα probe sequence (Fig. 25b). GABPα alone bound to the FPα sequence in a doublet, GABPβ as expected did not bind at all to the DNA whilst addition of both component peptides resulted in the formation of a single more retarded band. The GABPα doublet has been recognised to occur despite the presence of a single species of protein (313). It can sometimes occur due to two molecules of GABPα binding to two adjacent sites. When undifferentiated nuclear extract was added to the purified proteins, they formed the same set of complexes with complex doublet A having a mobility similar to that of complex doublet 1 (in Fig. 25a) and complex AB a lesser mobility, as in bands 2 and 3. Differences between Figs 25a and 25b related to varying extent of electrophoresis are apparent. Antibody co-incubations result in a supershifted band being formed in the presence of GABPα antibody with minimal reduction of both complexes A and AB, whilst GABPβ antibody prevents the formation of band AB without a resultant supershifted band and enhances band A formation. These results confirm that the changes seen in Fig.25a are specific effects of the antibodies on GABP proteins found in the NB4 nuclear extract and binding to FPα.

Fig. 25c shows that binding of purified GABP proteins to FPα is dependent on the core GGAA as is seen by the lack of binding to the mutated site. Comparing this with binding of nuclear proteins to the recognised mouse *neutrophil elastase* site shows FPα to be a strong GABP target site and confirms that the αβ species seen is indeed the dimer and not the tetramer (since there is only one ets site in this oligonucleotide).

8.2 Analysis of FPβ-bound proteins

8.2.1 Changes in binding to FPβ with differentiation

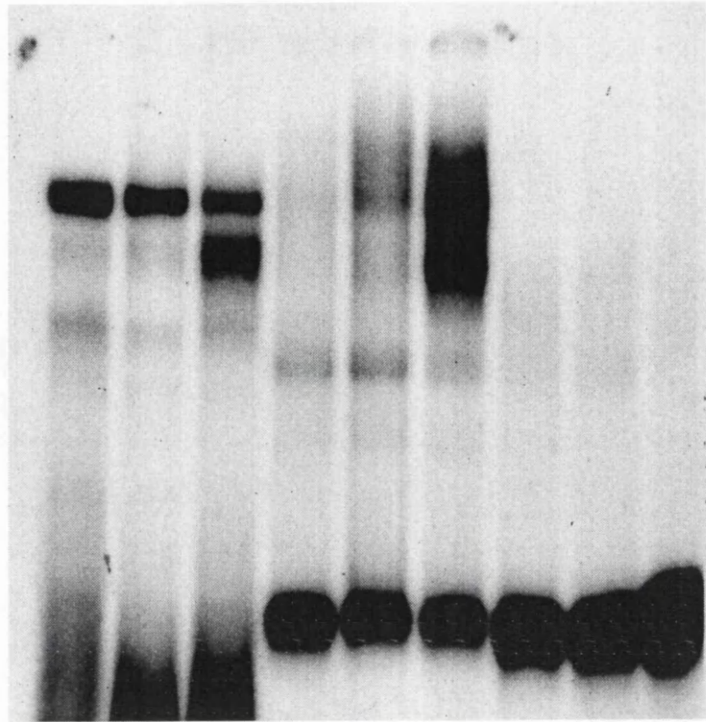
In order to study FPβ, a short sequence, -129/-104 was first used in experiments but when incubated with extracts from undifferentiated NB4 and from both differentiated lineages, the changes in protection identified during footprinting did not correlate with the pattern of bands seen. There was no increased binding with granulocytic as opposed to undifferentiated extract though some increase was detected with monocytic extract (Fig.26). For this reason, 10 base pairs of sequence were included on either end to include the whole of the protected sequence with 3-5 adjacent nucleotides on each side and in this case a clear increase in binding was seen with differentiated as opposed to undifferentiated extract. This binding, which is specific, is seen in Fig. 26 . It is interesting to note that despite rather similar FPβ footprints seen during footprinting experiments, monocytic and granulocytic extracts clearly contain different proteins that bind this site as can be seen from the different band patterns. One interesting quandary about Fig.26a is that inclusion of more FPβ flanking sequence resulted in the loss of a strong band seen with undifferentiated extract. One possibility may be that the extended oligonucleotide folds on itself and forms a tightly hybridised loop preventing easy access by the factors which bound the original shorter nucleotide.

Fig. 26b shows the effect of a number of mutations of potential transcription factor binding sites within FPβ. The different banding pattern seen is due to this EMSA being performed with different incubation conditions with the probe being added simultaneosly with the nuclear extract instead of after a 10 minute delay at 4°C. This

Fig.26 Changes in binding to FPβ with differentiation. (a) 5μg of nuclear protein from undifferentiated (U), granulocytic (G) or monocytic NB4 cells (M) was incubated with FPβ oligonucleotides of different lengths in buffer 1. Effective competition by a 200-fold molar excess of identical unlabelled FPβ oligonucleotide indicates that binding of protein from all three cell types (U*, G* and M*) on the longer FPβ oligonucleotide is specific.
(b) 5μg of nuclear protein from undifferentiated (U) or granulocytic (G) is incubated with wild type or mutated FPβ probes (indicated below the reaction lanes) in buffer 1. Addition of probe simultaneously with nuclear proteins, instead of after the usual 10 minute delay at 4°C, results in smaller protein complexes binding to the DNA whilst bigger protein-protein conglomerates do not form. X marks a band which appears on differentiation and which binds the myb site-mutated FPβ probe more strongly than the wild type FPβ but which cannot bind the probe mutated at the c/ebp or aml sites.

U G M U G M U* G* M*

a

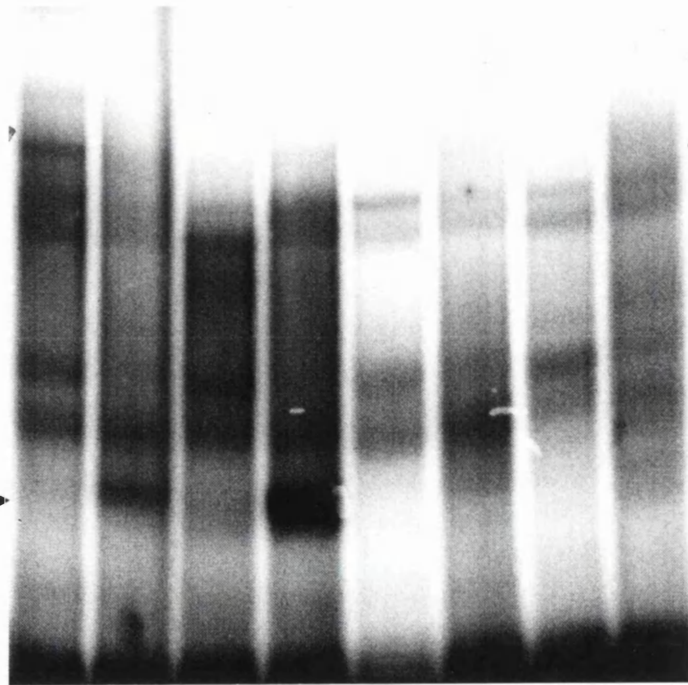


FPβ (-129/-104) FPβ (-140/-95)

U G U G U G U G

b

X →



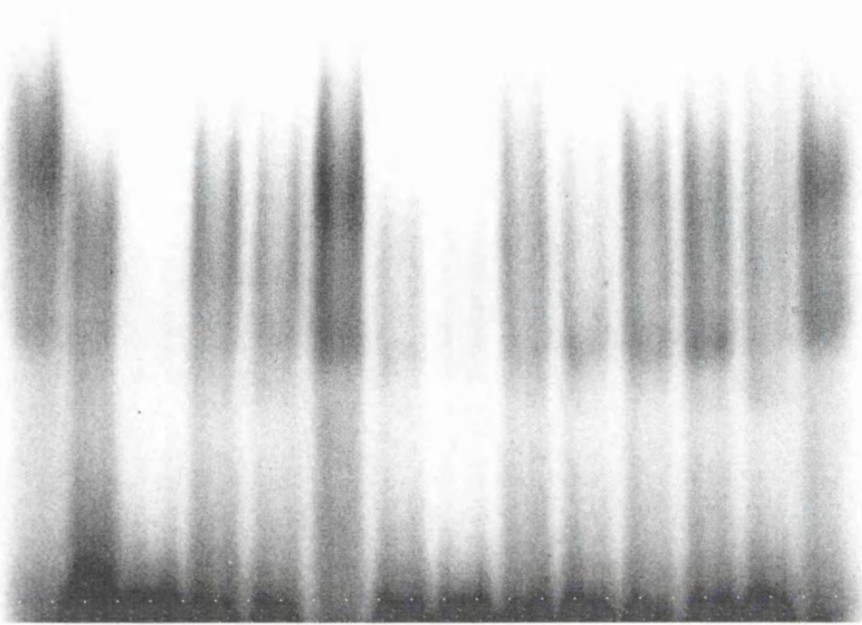
FPβ (-140/-95) FPβ -mut-myb FPβ -mut-c/ebp FPβ -mut-aml

Fig.27 Identification of FPβ binding proteins by competition EMSA.

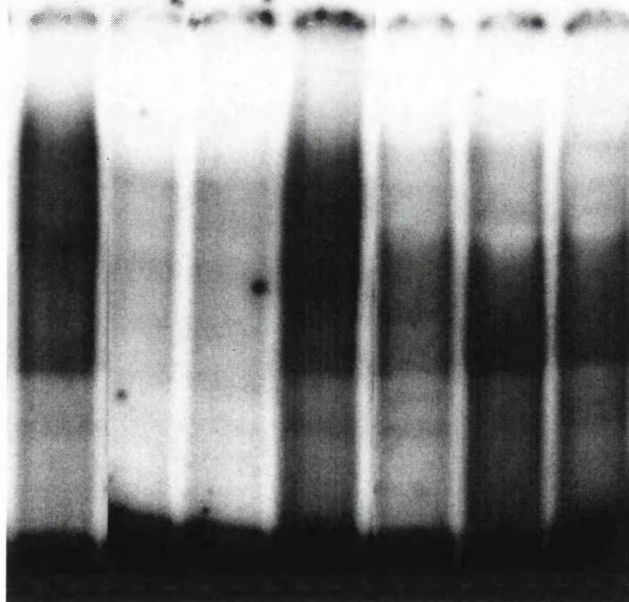
(a) 5μg of undifferentiated (UND) or granulocytic (DIFF) NB4 nuclear protein was incubated with a 200-fold molar excess of unlabelled competitor oligonucleotide (as shown) in buffer 2. [γ-^{32}p]-labelled FPβ probe was then added and the reaction is loaded onto a 4.5 % polyacrylamide gel and separated by electrophoresis.

(b) As in Fig.27a, 5μg of granulocytic NB4 nuclear protein was incubated with a 200-fold molar excess of unlabelled competitor oligonucleotide, however, in buffer 1. [γ-^{32}p]-labelled FPβ probe was added and the complexes formed separated by electrophoresis through a 6% polyacrylamide gel.

a



b



(Fig. 26b) where binding of band "X" to the myb site mutant is stronger than that to the unmutated FPβ probe. On the other hand, aml and particularly c/ebp site mutants are much poorer competitors of binding in the presence of granulocytic nuclear extract indicating the likely binding of these factors at this stage of differentiation. FPα, α1 and a short consensus c/ebp binding site from the *neutrophil elastase* promoter are all relatively poor competitors whilst FPγ is a slightly better competitor. Similar results were obtained with a different buffer system as seen in Fig. 27b where the myb site-mutated sequence is just as effective a competitor as wild type sequence and where c/ebp site mutated sequence is a much poorer competitor. Two different recognised binding sites for C/EBP from different promoters result in partial competition of the complex indicating that whilst C/EBP probably plays a major role in transcription factor binding to FPβ, it is not the only major player.

8.2.3 C/EBPε and GABPα bind to FPβ in granulocytic cells

In order to try and positively identify some of the factors binding to FPβ, antibodies were used to assess whether candidate transcription factors are present. The prime candidate for binding to this site was C/EBPε. Previous experiments had indicated a strong likelihood that a C/EBP family member was binding to this site. From work done by myself (unpublished observation) and others (370), it is apparent that C/EBPε is the main C/EBP family member expressed in NB4 cells whilst many other C/EBPs widely expressed in myeloid tissues and cell lines are absent.

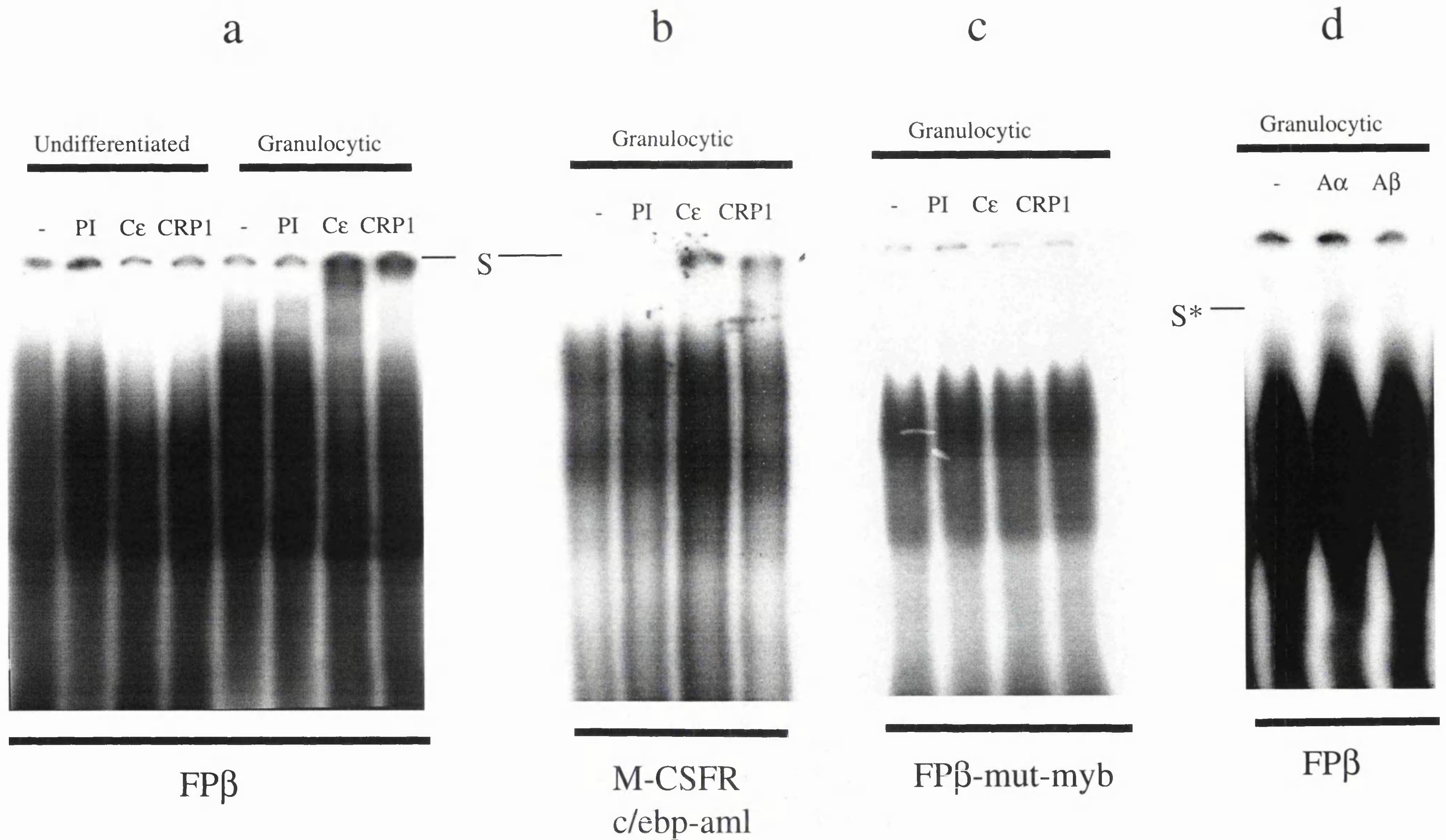
Indeed both an antibody directed against the N-terminal activation domain of human C/EBPε and an antibody raised against the C-terminal domain of the rat homologue of this protein, CRP1, resulted in the formation of strong supershifted bands when co-

incubated with granulocytic nuclear extract and labelled FPβ probe (Fig.28a). With undifferentiated NB4 nuclear protein, no such supershifted band was seen (probably due to the lesser abundance of C/EBP), although both antibodies, unlike pre-immune serum, disrupted some low mobility complexes, a feature also seen with differentiated extract. Similar supershifted bands were formed when these antibodies were incubated with granulocytic extract and a known binding site for C/EBPε, within the *m-csf receptor* promoter (Fig.28b). The antibodies resulted in no such supershift when incubated with FPβ mutated at the myb site (Fig 28c), suggesting that C/EBPε might require Myb to bind FPβ or that C/EBP bound to this probe might be inaccessible to the antibodies.

Due to the presence of a weak ets site within the FPβ probe sequence shifts using the anti-GABP antibodies were performed. As can be seen the GABPα antibody results in a shifted band (S*) not seen with the GABPβ antibody (Fig.28d). Similarly, studies with an anti-Myb monoclonal antibody, did not show any change in binding or supershift (not shown).

Therefore in summary both C/EBPε and GABPα may bind to FPβ in granulocytic NB4 cells (Fig.28). C/EBPε and AML-1 appear to bind FPβ more strongly in differentiated cells (Fig.26b) whilst Myb appears more important for FPβ protein binding in undifferentiated cells (Fig.23a). However, in the presence of granulocytic cell extract, whilst mutation of the myb site results in stronger FPβ binding (as noted by competition experiments with mutated FPβ sites), any C/EBPε bound is inaccessible to antibodies suggesting a possible conformational change in the protein or an interaction which inhibits antibody access.

Fig.28 Identification of FPβ-binding proteins by means of antibody supershifts.
(a), (b) and (c) 5μg of undifferentiated or granulocytic NB4 nuclear protein (as indicated) was incubated with [γ-^{32}p]-labelled FPβ, M-CSFR (c/ebp-aml site) or FPβ-mut-myb probes respectively in buffer 2. No antibody (-), pre-immune serum (PI), anti-N-terminal C/EBPε antibody (Cε), or anti-rat -C-terminal C/EBPε antibody (CRP1) are co-incubated with the above reactions (as indicated) and the complexes formed separated by electrophoresis through a 4.5% polyacrylamide gel.
(d) 5μg of granulocytic NB4 nuclear protein was incubated with [γ-^{32}p]-labelled FPβ in buffer 3. No antibody, (-), anti-GABPα antibody (Aα) or anti-GABPβ antibody (Aβ) was co-incubated and the complexes formed separated by electrophoresis through a 4.5% polyacrylamide gel.



a
b
c
d
Undifferentiated
Granulocytic
- PI Cε CRP1 - PI Cε CRP1
S
Granulocytic
- PI Cε CRP1
Granulocytic
- PI Cε CRP1
Granulocytic
- Aα Aβ
S*
FPβ
M-CSFR
c/ebp-aml
FPβ-mut-myb
FPβ

8.3 Analysis of FPγ-bound proteins

8.3.1 Changes in FPγ binding with differentiation

Despite the relatively small size of FPγ and the relatively weak footprinting, computer analysis of the sequence indicated a rich abundance of strong potential transcription factor binding sites, including a good pu.1 consensus site and adjacent, overlapping c/ebp and aml sites (see Fig. 17).

Fig. 29 shows clear changes in binding to this site with differentiation. The appearance of a low mobility (large) complex (band X) and an even clearer high mobility doublet (bands 3,4) with granulocytic differentiation show that this site may be functionally important in regards to changes in *defensin* expression with differentiation. Mutation of the core ets GGAA sequence within the pu.1 site results in loss of both these granulocytic- specific bands and the appearance of a low mobility doublet which is uninfluenced by lineage or differentiation. There is also a marked enhancement of the intermediate mobility complex (marked as 2) which again is relatively invariable with differentiation. The mutation study suggests that the granulocytic-specific high mobility complexes may contain Ets factors.

8.3.2 Characterisation of FPγ binding by competition EMSAs

Due to the clear band pattern seen with FPγ on EMSA, (possibly due to its much smaller size than FPs α and β), competition EMSAs were very informative in the sense that very clear patterns could be obtained so a good number of experiments were performed. As seen in Fig. 30a, the larger (lower mobility) complexes 1 and/or 2 have a

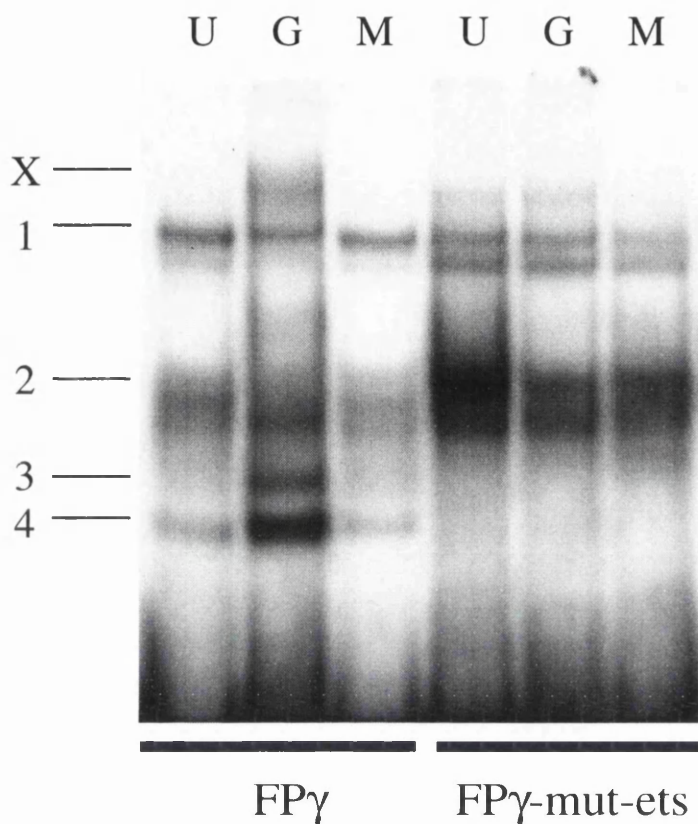


Figure 29. Changes in binding with differentiation to FPγ and to ets-site mutated FPγ.
U, G, and M refer to undifferentiated, granulocytic and monocytic nuclear extract (5 μg) respectively. Whilst the high mobility complexes seen as the band doublet 3 and 4 were reproducibly seen, the band X was often indistinguishable from band 1 in other experiments.

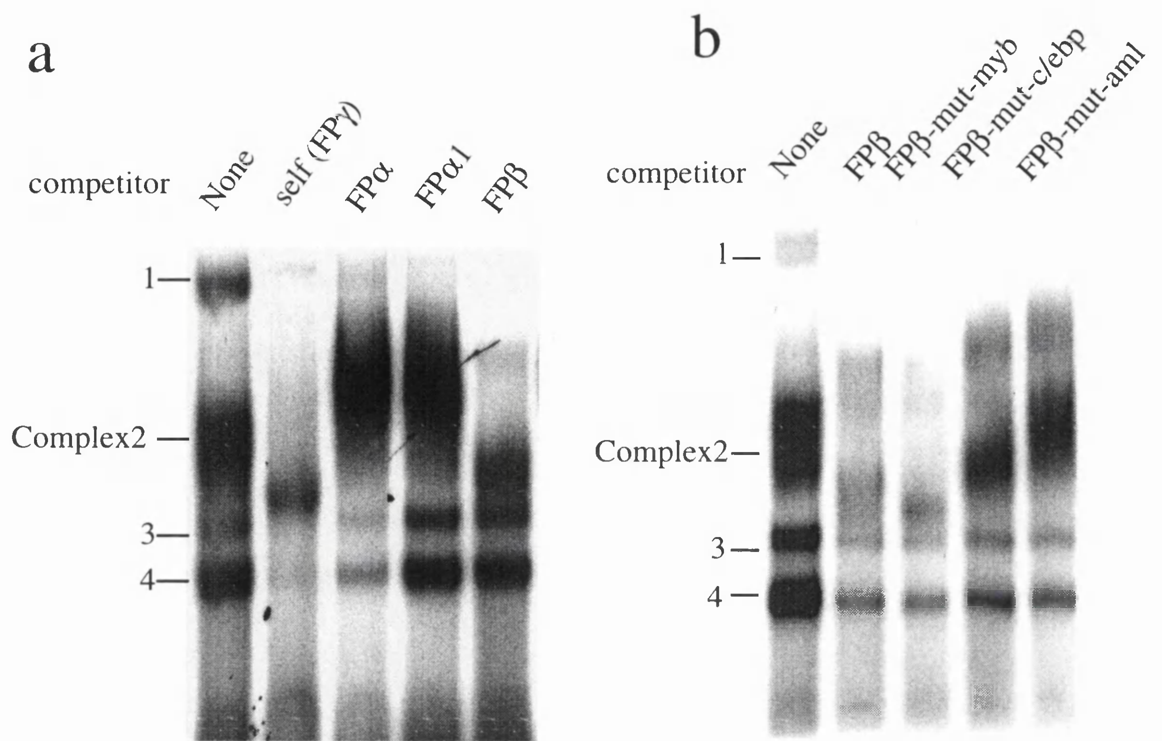
non-specific component which still binds after competition with 200-fold molar excess of unlabelled FPγ. FPα is a weak competitor though it does compete the lower band doublet slightly which is not surprising considering that they are probably Ets factors. FPα1 cannot compete much except for a component of complex 1 (possibly a non-specific binding protein since a loss of part of complex 1 and a consequent enhancement of complex 2 occurs with many different competing oligonucleotides).

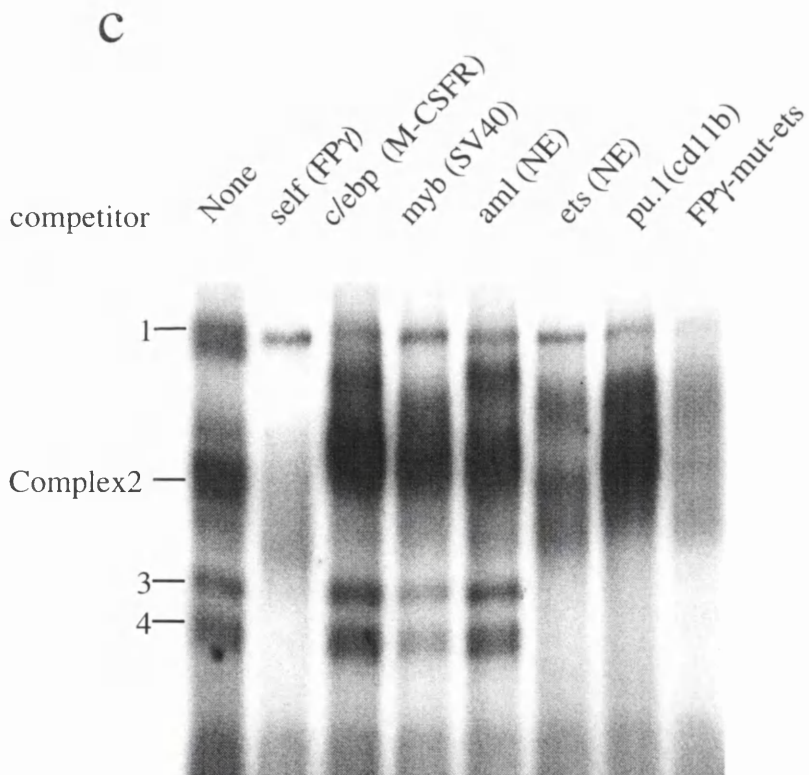
FPβ on the other hand, is a good competitor for some of the components of complexes 1 and 2 whilst not having any effect on the binding of the high mobility complexes (band doublet 3 and 4).

Competition EMSAs using site-specific mutant FPβ oligonucleotides (Fig.30b) show that whilst wild type and myb site mutants are strong competitors of the complex 1 and 2 proteins, the c/ebp and aml mutants are incapable of such competition. This indicates that these two transcription factors are bound to the FPγ sequence as was suggested by computer analysis.

Fig. 30c shows competition experiments using a number of recognised binding sites for different transcription factors thought to be binding to this site as oligonucleotide competitors. As noted with other oligonucleotide competitors, complex 1 is disrupted to some extent by all, possibly due to competing off some non-specific component. Sites known to bind C/EBP, Myb and AML-1 do not bind the residual proteins from the lower mobility complexes, nor do they bind the high mobility doublet though some slight competition does appear to occur in the presence of the myb site. The Ets binding sites from the *cd11b* promoter (which binds PU.1) and the *neutrophil elastase* promoter (which binds GABP or PU.1) both efficiently compete away the Ets factor-related complexes bands 3 and 4. It is interesting to note that FPγ mutated at the Ets binding site is also capable of competing bands 3,4. This suggests that while such an

Fig. 30 Identification of proteins binding to FPγ by competition EMSAs.
(a), (b) and (c) 5μg of granulocytic NB4 nuclear protein was incubated with a 200-fold molar excess of unlabelled competitor oligonucleotide, (shown) in buffer 1. [γ-^{32}p]-labelled FPγ is then added and the complexes formed separated by electrophoresis through a 6% polyacrylamide gel. Self competition shows that protein binding to FPγ is specific.


a
competitor
None
self (FPγ)
FPα
FPα1
FPβ
1
Complex2
3
4
b
competitor
None
FPβ
FPβ-mut-myb
FPβ-mut-c/ebp
FPβ-mut-aml
1
Complex2
3
4


c
competitor
None
self (FPγ)
c/ebp (M-CSFR)
myb (SV40)
aml (NE)
ets (NE)
pu.1(cd11b)
FPγ-mut-ets
1
Complex2
3
4

oligonucleotide is incapable of binding the Ets proteins of these complexes directly, it is capable of binding them as part of a complex with other factors by means of protein-protein interactions. Amongst the Ets-binding sites, there are clear differences with regards to the competition of lower mobility complexes. Whilst the *neutrophil elastase* site competes for the binding of some complex 2 proteins, the *cd11b* site does not. This suggests that Ets factors other than PU.1 may also form part of the lower mobility complexes.

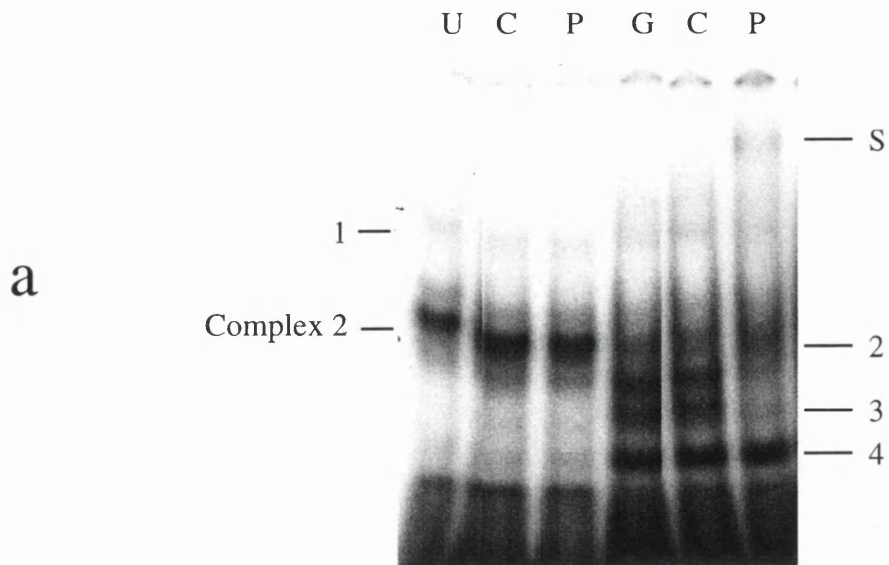
8.3.3 PU.1 binds to FPγ

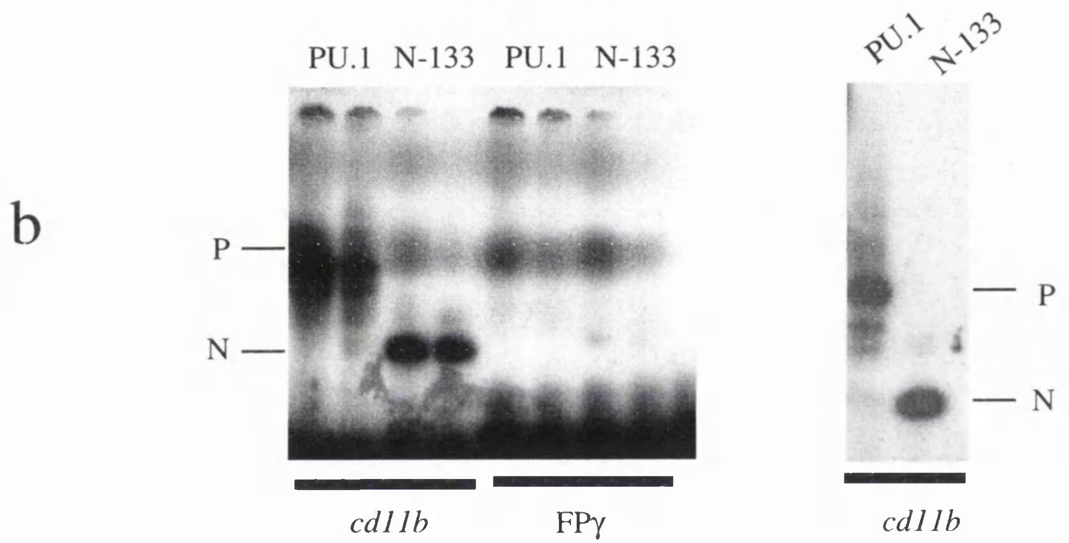
In order to identify the Ets factors binding to FPγ, antibody studies were performed (Fig. 31 a, c). The main candidate was PU.1 both from computer analysis of the site and from the presence of the characteristic doublet (bands 3 and 4) which is often seen in myeloid cells, with PU.1-binding sites (322).

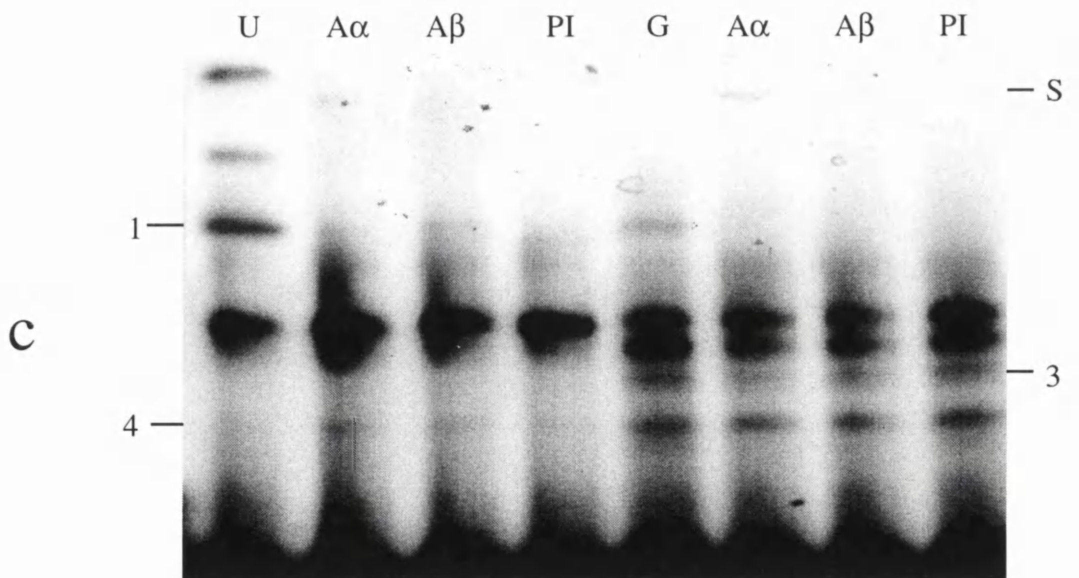
Co-incubation of a monoclonal antibody against PU.1 with the EMSA reaction (Fig.31a) resulted in the supershifting of band 3 (but not band 4) and the consequent formation of a supershifted complex (band S). This pattern of supershift is again characteristic of PU.1 with the higher mobility band 4 representing a proteolytic breakdown product of PU.1 that can bind DNA but is unrecognised by most antibodies (322).

Using a transcription/translation system (Promega), both *in vitro* translated PU.1 and a deletion mutant (N-133) lacking the N-terminal 133 aa residues (which include the activation domains) are capable of strongly binding the PU.1 binding site of the *cd11b* promoter. They also bind to the *defensin* promoter site FPγ (though much less strongly). Some diffuse bands are seen due to ^{35}S-induced non-specific labelling of

Fig.31 Ets protein binding to FPγ.
(a) 5μg of undifferentiated (U), or granulocytic (G) nuclear protein extract was incubated with [γ-^{32}p]-labelled FPγ in the presence of buffer 3. No antibody (U or G), control monoclonal antibody against phospholipase A_2 (C), or anti-PU.1 monoclonal antibody (P), co-were incubated and the complexes formed, separated by electrophoresis through a 6% polyacrylamide gel. Bands 1-4 identify complexes migrating in a similar pattern to those seen with buffer 1, (Fig29,30). A supershifted complex formed upon co-incubation with PU.1 antibody is marked as S.
(b) Both left and right panels show in vitro translated PU.1 or N-133 (an N-terminal PU.1 deletion mutant), co-incubated with the *cd11b* or FPγ ets site labelled probes, in buffer 3. In the left panel, diffuse bands are due to non-specific labelling of proteins during the in vitro translation reaction by ^{35}S. (This was excluded from the reaction to produce proteins used in the EMSA shown in the right panel.) These diffuse bands obscure the band due to PU.1 binding to FPγ yet a band due to N-133 binding is visible.
(c) 5μg of undifferentiated (U), or granulocytic (G) nuclear protein extract was incubated with [γ-^{32}p]-labelled FPγ in the presence of buffer 3. Antibodies against GABPα (Aα) or against GABPβ (Aβ) or pre-immune serum (PI), were added to the incubations and the complexes formed, separated by electrophoresis through a 6% polyacrylamide gel. Bands 1, 3 and 4 refer to the same complexes as those seen in Fig 31(a) whilst S indicates a supershifted complex formed with the GABPα antibody. Two bands of lower mobility than band 1, seen in the lane labelled "U" were not seen in Fig 31 (a) nor in any other incubations with FPγ in this or any other buffers and were therefore considered spurious.


U C P G C P
a
1
Complex 2
S
2
3
4


b
PU.1 N-133 PU.1 N-133
P
N
cd11b
FPγ
PU.1
N-133
P
N
cd11b


U Aα Aβ PI G Aα Aβ PI
S
1
c
3
4

translation mix components and non-specific binding of transcription/translation mix factors to the probe DNA (Fig. 31b).

Antibodies against GABPα also showed a supershift with both undifferentiated and granulocytic nuclear extract. Two spurious accessory bands can be seen here with undifferentiated NB4 extract, which have a lower mobility than band 1. The intensity of band 1 is reduced by anti-GABPα antibody more than by anti-GABPβ antibody. Concomitant with this a supershifted band (S) appears, as does a band of similar mobility to band 4. One explanation may be that some of the GABPα may have been displaced from the DNA (band 1 without the antibody seems more intense than the sum of the residual band 1 and the supershifted band.) The PU.1 proteolytic product can therefore bind to FPγ DNA in its place (see section 16.3).

When incubated with granulocytic extract, GABPα antibody induced the appearance of a similar supershifted band and a concomitant reduction of band 3. This band is likely to be due to PU.1 so it is unlikely to be shifted by the GABPα antibody. A more likely explanation is that GABPα formed part of complex (not necessarily DNA-bound) and this was partly disrupted by the antibody. PU.1 being an alternate Ets transcription factor may then interact with the other factors in this low mobility complex, through its ETS domain, resulting in less binding of the PU.1 to DNA by itself.

The PU.1 proteolytic product seen as band 4 is probably incapable of substituting GABPα since protein interaction domains other than the pure DNA binding domain may be necessary. The need for domains outside the ETS domain in co-operative interactions is confirmed in transfection experiments (see section 13.4).

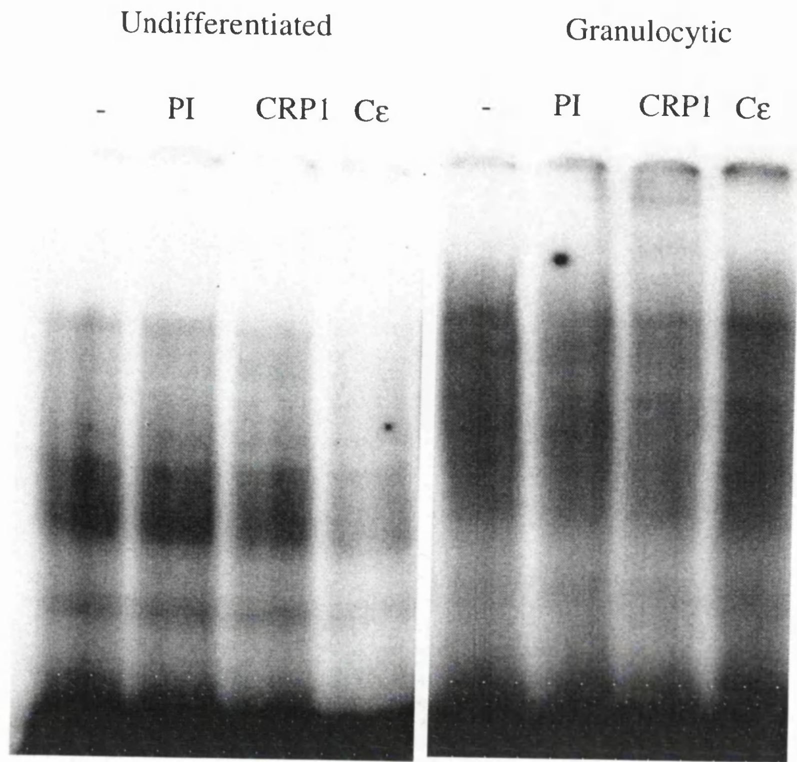
8.3.4 C/EBPε binds to FPγ as well as to FPα

Since there is considerable competition between FPβ and FPγ and since c/ebp site mutation inhibits this cross competition between these two sequences, I decided to perform tests to see whether C/EBPε is binding to FPγ. Similarly FPβ is capable of competing FPα binding, a feature which is also dependent on an intact c/ebp site. Co-incubations of anti-C/EBPε antibodies with undifferentiated and differentiated NB4 extracts and with FPα or FPγ probes are shown in Fig. 32a and b respectively.

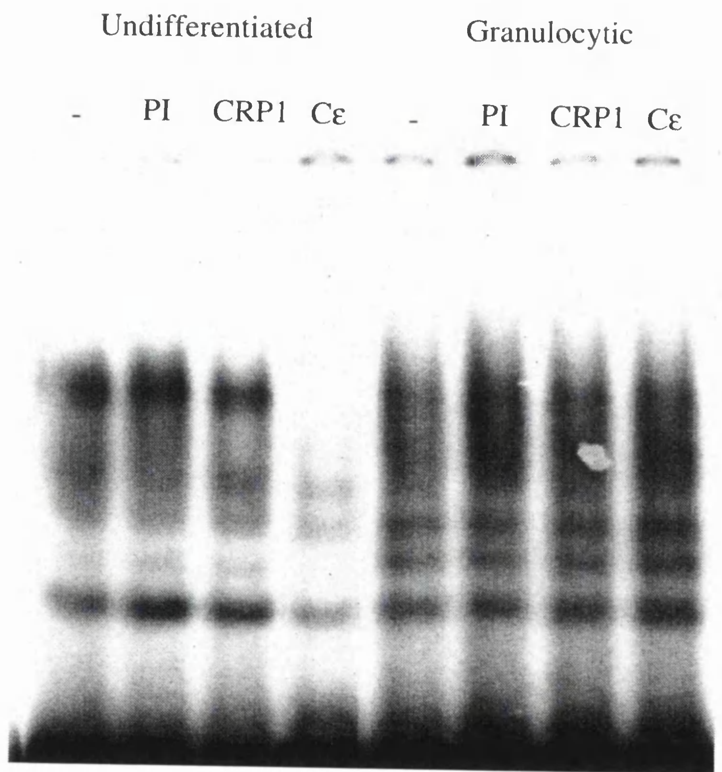
Though it is clearer in the case of FPγ, in both cases a similar pattern emerges. In EMSAs using undifferentiated extract, the anti-N-terminal antibody named Cε disrupts binding of a number of complexes unlike the anti-C-terminal antibody named CRP1. No supershifted complex is seen with either antibody. When differentiated nuclear extract is used, no such supershift or disruption of complexes is seen to occur. This result is unexpected because both from previously published studies, as well as from my own studies with FPβ; it is apparent that more C/EBPε is found in myeloid cells as they are differentiated along the granulocytic pathway. Therefore in this differentiated state, it is not that C/EBPε is not present in the extract but either that it is not bound to DNA, or that it is bound to DNA in a manner such that it is not accessible to the antibodies. In differentiated extract EMSA, other transcription factors (not present in undifferentiated extract), may bind to the DNA and in so doing, prohibit C/EBPε binding. In the case of FPα, changes in binding of GABPβ (upon differentiation) may be involved whilst enhanced PU.1 binding may be responsible in the case of FPγ. Why the anti-C-terminal antibody has no effect is unclear but it would suggest that the C-terminal part of the protein is tightly complexed to other proteins or to DNA in such a manner as to prevent antibody binding. On the other hand antibody binding to the

Fig.32 C/EBPε binding to FPα and FPγ.
(a) 5μg of undifferentiated, or granulocytic nuclear protein extract (as indicated) was incubated in the presence of buffer 2 with [γ-^{32}p]-labelled FPγ. Pre-immune serum (PI), anti-rat -C-terminal C/EBPε antibody (CRP1) or anti-N-terminal C/EBPε antibody (Cε), are co-incubated with the above reactions (as indicated) and the complexes formed separated by electrophoresis through a 4.5% polyacrylamide gel. When using such electrophoresis conditions protein binding to FPα is weak resulting in indistinct and smeared bands.
(b) Reactions are prepared and separated exactly as described above, substituting labelled FPγ for FPα, as probe.

a



b



N-terminal part of C/EBPε results in disruption of some complexes suggesting that this part of the protein may be involved in some protein interactions which are not however strong enough to prevent the interaction with antibody.

Chapter 9 : Factor interactions on the *defensin* promoter

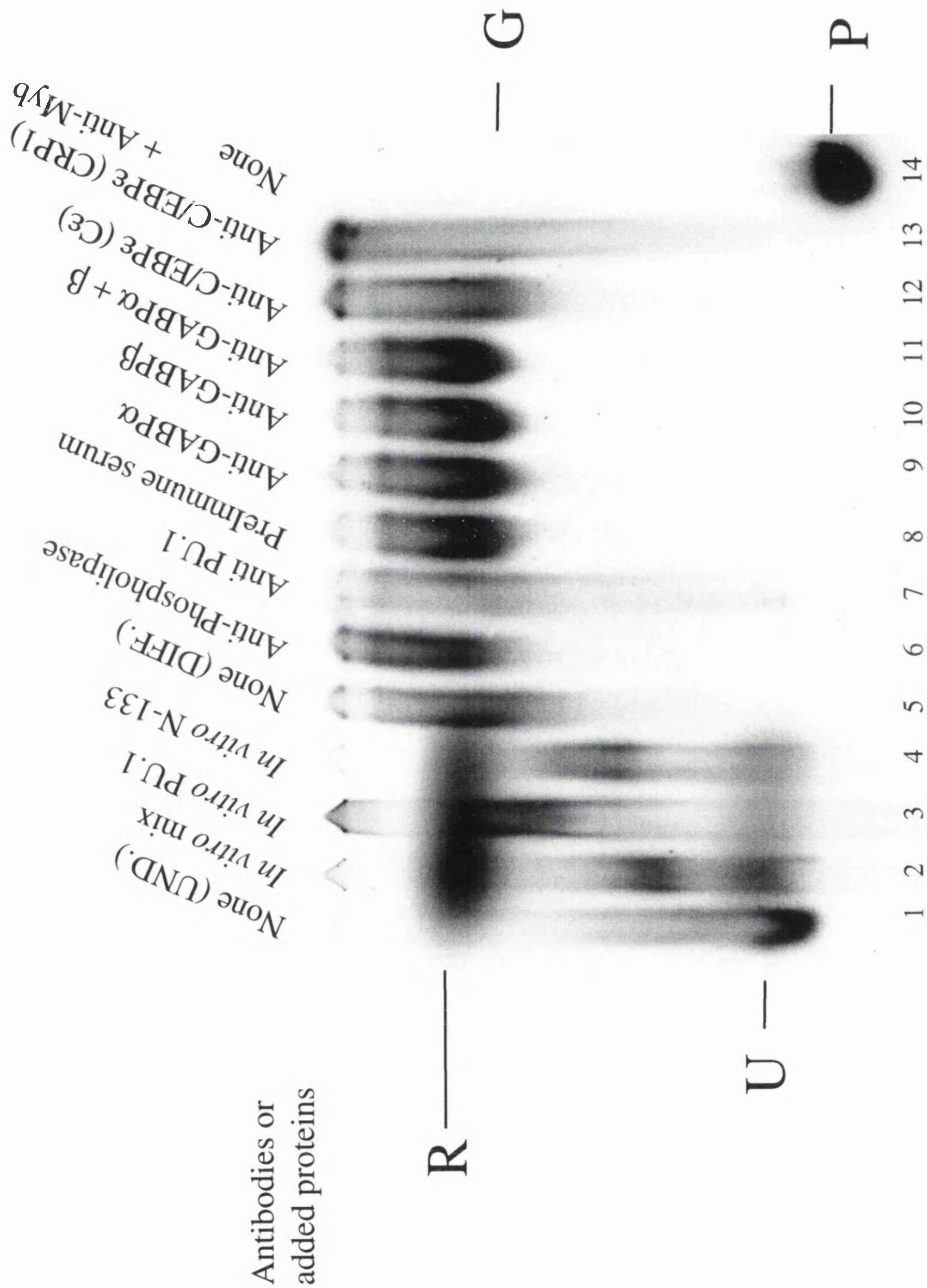
Both footprinting experiments and certain EMSAs indicated that different factors bound to sequences within the *defensin* promoter may also interact with each other thereby influencing their capability to bind DNA and to form complexes on the promoter sequences. This was investigated in more detail.

9.1 Antibodies alter NB4 nuclear protein binding to the *defensin* promoter

Using specific transcription factor binding sites as competing sequences, it had been impossible to detect which factors if any, play an essential role in the granulocytic complex formed on the -240/+15 *defensin* promoter sequence (see Fig.21). Evidence had been obtained using antibodies about different factors binding to sites within the promoter. Therefore, these antibodies were co-incubated in EMSAs with the whole promoter sequence labelled as probe, to see whether they had any effect on the promoter complex. In this study, a different buffer system was used which distinguishes between the complexes formed by undifferentiated and granulocytic NB4 extract (lanes 1 and 5 respectively in Fig. 33). Using this buffer, monocytic extract produces a band complex of similar mobility to granulocytic extract (not shown) so that the main factors binding the DNA in this buffer are likely to be those common among monocytic and granulocytic extracts. These are indeed many as can be seen in the footprinting experiments. In this EMSA, antibodies were pre-incubated with the nuclear extract for

Fig.33 Identification of proteins binding to the –240/+15 defensin upstream sequence.

5μg of undifferentiated (UND), or granulocytic (DIFF) NB4 nuclear protein extract was incubated with in vitro translated proteins or translation mix, (shown) or with various antibodies (shown) in the presence of buffer 1. Labelled –240/+15 defensin probe sequence was added, and the complexes formed, separated by electrophoresis through a 4% polyacrylamide gel. P,U, and G identify the probe alone, the complex formed with undifferentiated extract and that formed with differentiated extract respectively. R is a band caused by ^{35}S-radioactively labelled proteins in the translation reaction.



Antibodies or
added proteins
None (UND.)
In vitro mix
In vitro PU.1
In vitro N-133
None (DIFF.)
Anti-Phospholipase
Anti PU.1
PreImmune serum
Anti-GABPα
Anti-GABPβ
Anti-GABPα + β
Anti-C/EBPε (Cε)
Anti-C/EBPε (CRP1)
None + Anti-Myb
R
U
G
P
1 2 3 4 5 6 7 8 9 10 11 12 13 14

a couple of hours prior to adding in the probe unlike other regular EMSAs where nuclear extract and antibodies are added one after the other and probe is added after a further 10 minutes.

Lanes 2 to 4 show the effects of adding *in vitro* translated PU.1 to undifferentiated extract. Undifferentiated protein extract alone is shown in lane 1. The complex formed is marked as band U. PU.1 (lane 3) reduces the mobility of this complex to an extent similar to that of the granulocytic extract complex (marked as G). This suggests that PU.1 is a major difference between the two states of differentiation, as indicated also by FPγ EMSA experiments. As can be seen in lanes 2 and 4 respectively, addition of the *in vitro* transcription/translation mix alone, or of the N-133 deletion mutant of PU.1 synthesised in this mix did not have such an effect. Non-specific bands are seen across all these three lanes as a result of radioactivity due to the ^{35}S in the synthetic reaction - the stronger of these marked as R.

A monoclonal antibody against PU.1 (lane 7) had a complementary yet opposite effect when incubated with granulocytic extract. The formation of the granulocytic complex was inhibited, suggesting that PU.1 is indeed important for transcription factor complex formation on the *defensin* promoter at this stage of differentiation. A control monoclonal antibody had no such effect. Anti-C/EBPε antibody (CRP1) in the presence of anti-Myb antibody also disrupted the complex indicating the importance of these proteins in granulocytic factor promoter complexes and again suggesting that in the case of C/EBPε co-operative binding may occur between Myb and C/EBP.

Anti-C/EBPε antibody alone had no apparent effect. Antibodies against GABPα or GABPβ also had no specific effect. A slight reduction in mobility (supershift) seen with these and other antibodies including non-specific controls or pre-immune serum suggests some non-specific serum binding to promoter-bound factors.

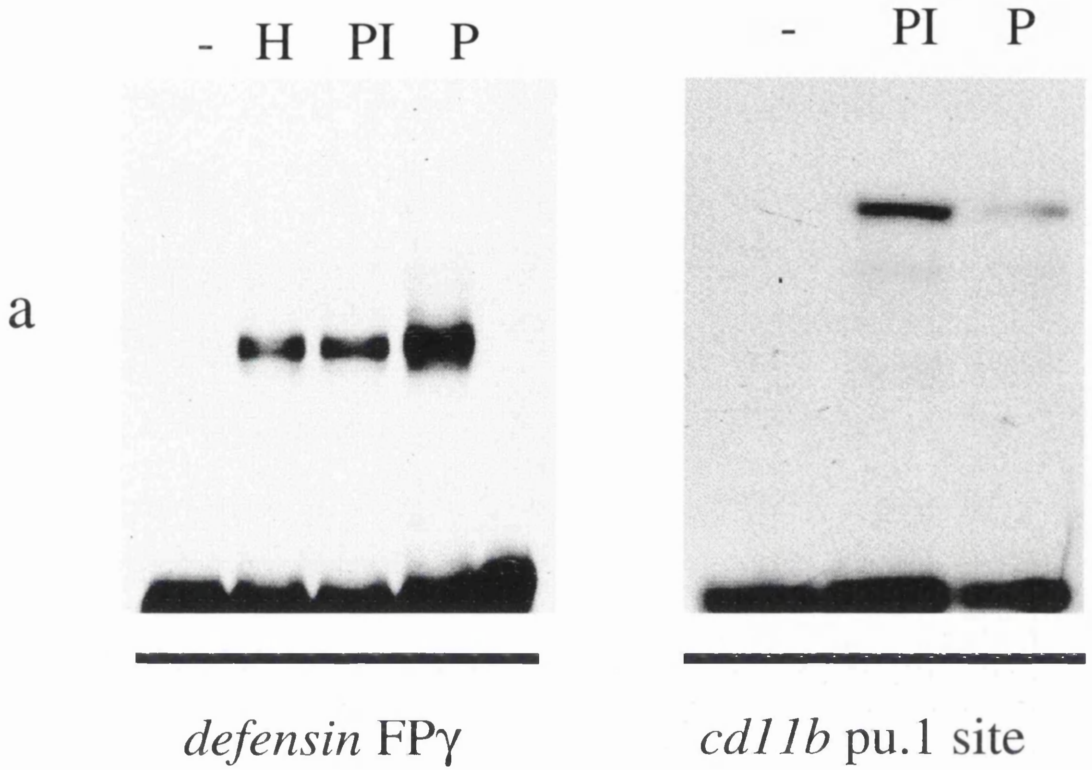
9.2 Anti-Ets factor antibodies increase HL60 nuclear factor binding to FPγ

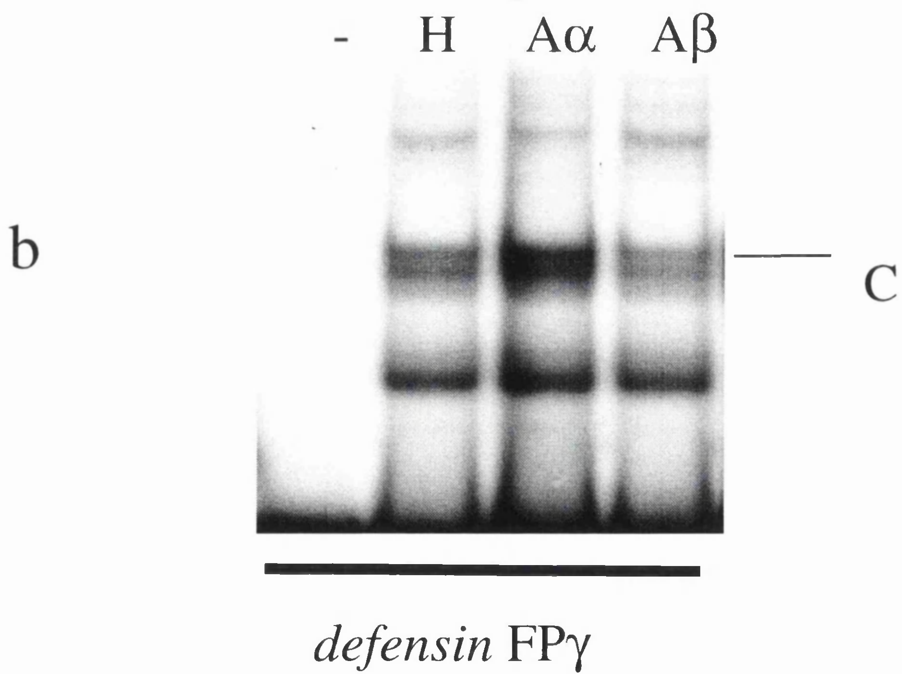
As well as some initial studies with FPα using NB4 extracts, previous results obtained by another researcher in our laboratory, Thomas Jamieson, also indicated interactions between different factors. When using HL60 nuclear extract to perform EMSA experiments, on the *defensin* promoter, it was noted that anti-PU.1 antibody (in this case a polyclonal preparation provided by C.K.Glas (Beth Israel Hospital, Haarvard Medical School, Boston, MA) enhanced the formation of the predominant complex seen bound to FPγ (Fig. 34a). Pre-immune serum had no such effect. When performing similar experiments with the same antibodies on a recognised PU.1-binding site from the *cd11b* promoter, the same anti-PU.1 antibody inhibited the formation of the complex suggesting that it did indeed bind PU.1, possibly close to, or at the ets site. Anti-GABP antibodies were also co-incubated with HL60 nuclear extract and labelled FPγ DNA (Fig. 34b). GABPα antibody also had a clear enhancing effect on the predominant complex marked as C (it is interesting to note that the PU.1 doublet seen clearly with NB4 extract is absent here with only the proteolytic form visible). GABPβ antibody had no such effect or if anything reduced the complex formation slightly. It is important to point out that the much less complex banding pattern seen with HL60 extracts, in Fig.34a is due to the extract being prepared in a simpler and more rapid way. The extracts used in Fig.34b were prepared by a method similar to that used in the other EMSA and footprinting experiments and which were purposely designed to isolate transcription factors.

Fig.34 Increased binding of HL60 proteins to FPγ upon co-incubation with anti-Ets factor antibodies.

(a) 5μg of HL60 nuclear protein extract (H) was incubated with [γ-^{32}p]-labelled FPγ or the *cd11b* ets probe (as shown) in the presence of buffer 1. Pre-immune serum (PI) or polyclonal anti-PU.1 antibody (P) was added to the incubation reaction and the complexes formed, separated through a 6% polyacrylamide gel.

(b) μg of HL60 nuclear protein extract (H) was incubated with [γ-^{32}p]-labelled FPγ in buffer 1. GABPα antibody (Aα) or GABPβ antibody (Aβ) were co-incubated with these reactions and the complexes formed separated by electrophoresis through a 6% polyacrylamide gel. The complex whose binding is increased by GABPα antibody is marked as C.


- H PI P
- PI P
a
defensin FPγ
cd11b pu.1 site


- H Aα Aβ
b
C
defensin FPγ

Therefore a similar pattern is observed where there is increased binding of a particular protein (or proteins) to FPγ when anti-Ets factor antibodies are co-incubated. No supershifted bands are seen, so this suggests a picture where the antibodies are interacting with their respective Ets factors and disrupting a complex formed in solution between the Ets factor and another factor. (Such DNA-independent interactions are known to occur between Ets factors and other transcription factors including Notch-like GABPβ and also C/EBP α,β(239;379)). Disruption of this complex allows the other factor to bind to the DNA target site thus enhancing the intensity of a previous band or resulting in the formation of new complexes. In order to further test this hypothesis, we performed similar experiments on NB4 cells to try and replicate this phenomenon primarily using FPα as our sequence of interest and trying to identify the factor responsible for the enhanced binding upon Ets factor removal.

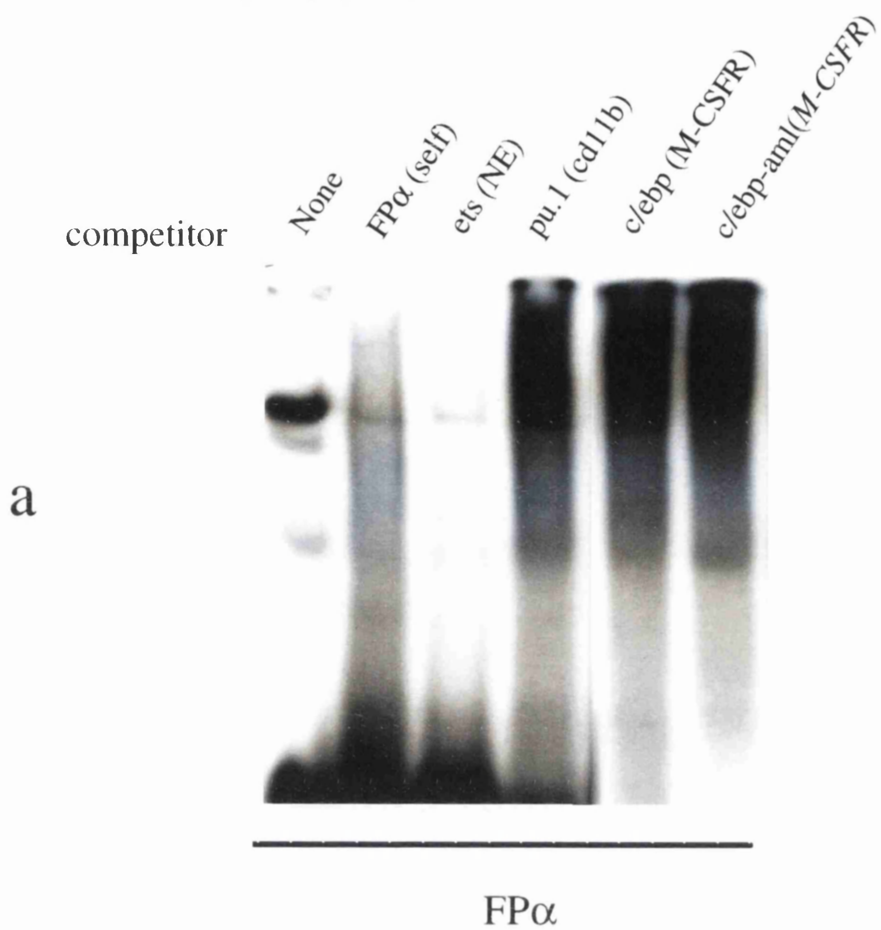
9.3 Increased NB4 nuclear protein binding to FPα upon competition

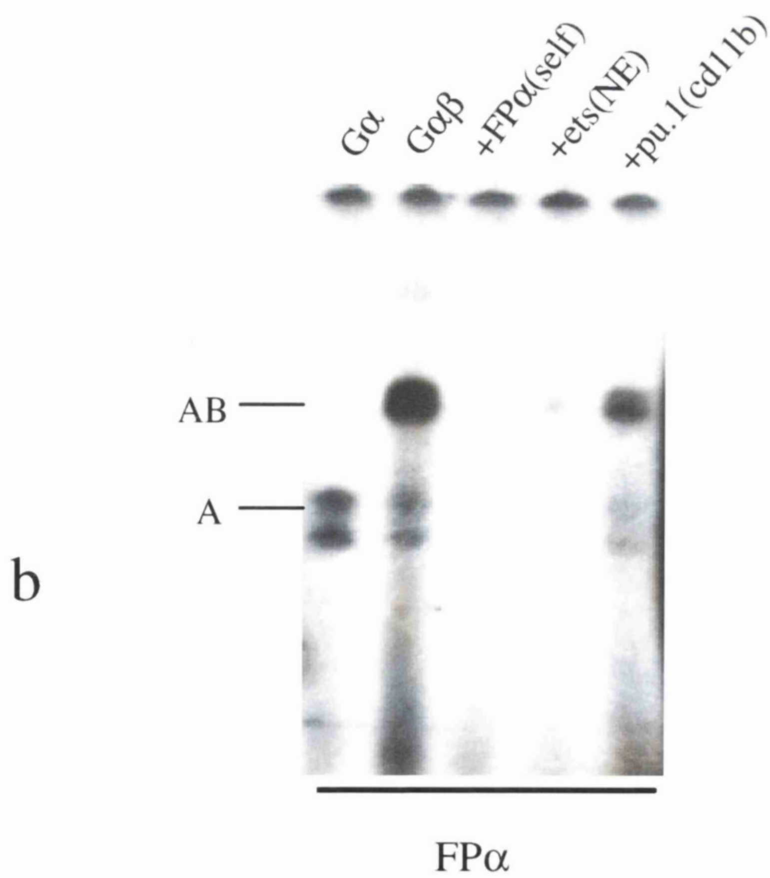
9.3.1 Low mobility complex binding after prolonged competition

Using FPα as a labelled probe, granulocytic nuclear extract was incubated with several competing oligonucleotides (Fig.35a). The incubation was allowed to proceed overnight at 4°C prior to running out on the gel. As can be seen this allows much clearer complexes to be formed on the long FPα probe than had been previously seen. A 200-fold molar excess of unlabelled FPα as well as of competitor Ets-binding site from the *neutrophil elastase* (NE) promoter is able to compete away most complexes bound to this site. However, the pu.1 binding site from the *cd11b* promoter as well as c/ebp binding sites from the M-CSF receptor promoter resulted in a marked increase in

Fig. 35 More FPα competition EMSAs. (a) Enhanced binding of NB4 nuclear proteins to FPα following a prolonged incubation with competing oligonucleotides. 5μg of granulocytic NB4 nuclear protein extract was incubated with 200-fold molar excess of unlabelled competitor oligonucleotide, (shown) and with [γ-^{32}p]-labelled FPα in buffer 1. Incubations were allowed to proceed for 16 hours slowly rotating at 4°C. The complexes formed were then separated by electrophoresis through a 6% polyacrylamide gel.

(b) Assessing the binding specificity of different ets site competitor oligonucleotides. Purified GABPα(Gα), GABPα together with GABPβ(Gαβ), or both sub-units together with 200-fold molar excess of different unlabelled competitor oligonucleotides (shown), were incubated together with [γ-^{32}p]-labelled FPα in buffer 3. The complexes formed were then separated by electrophoresis through a 4.5% polyacrylamide gel.


competitor
None
FPα (self)
ets (NE)
pu.1 (cd11b)
c/ebp (M-CSFR)
c/ebp-aml(M-CSFR)
a
FPα


Gα
Gαβ
+FPα(self)
+ets(NE)
+pu.1(cd11b)
AB
A
b
FPα

binding. Fig.35b shows that whilst the NE ets site strongly competes for GABP bound to FPα, the *cd11b* ets site is a much weaker competitor. This suggests that removal of one factor (in this case by binding to its recognised DNA site) allow other components to bind to FPα (Fig 35a). The increase in binding seen here is greater than that seen during shorter incubations and results in a markedly retarded complex. This was hypothesised to be due to the binding of a particular transcription factor to FPα in the absence of competing factors which could then recruit other transcription factors (or components of the transcription machinery) to FPα. In order to maintain some uniformity (despite certain benefits of a longer incubation), the standard shorter incubation was used for all other experiments

9.3.2 Increased FPα binding after competion with Ets-binding oligonucleotides is due to C/EBPε

Standard co-incubations with undifferentiated NB4 nuclear extract, radiolabelled FPα probe and various competitor oligonucleotides, were performed (Fig.36a). Different patterns of increased binding were detected.Oligonucleotides which bind C/EBP factors such as FPβ, FPα1 (potential if not proven binding), and other recognised C/EBP binding sites resulted in a marked increase in the formation of high mobility (low molecular weight) complexes which were seen in a number of discreet bands (marked as H). At the same time the lower mobility (higher molecular weight) bands (marked as L) are reduced in intensity to a greater or lesser extent (lane 2) or become more distinct (lanes 3, 8 and 9).When Ets-binding oligonucleotide competitors (such as FPγ, the PU.1 binding site of *cd11b*, or a short part of FPα which includes just the core GABP-binding ets site but not adjacent c/ebp or aml sites) are co-incubated

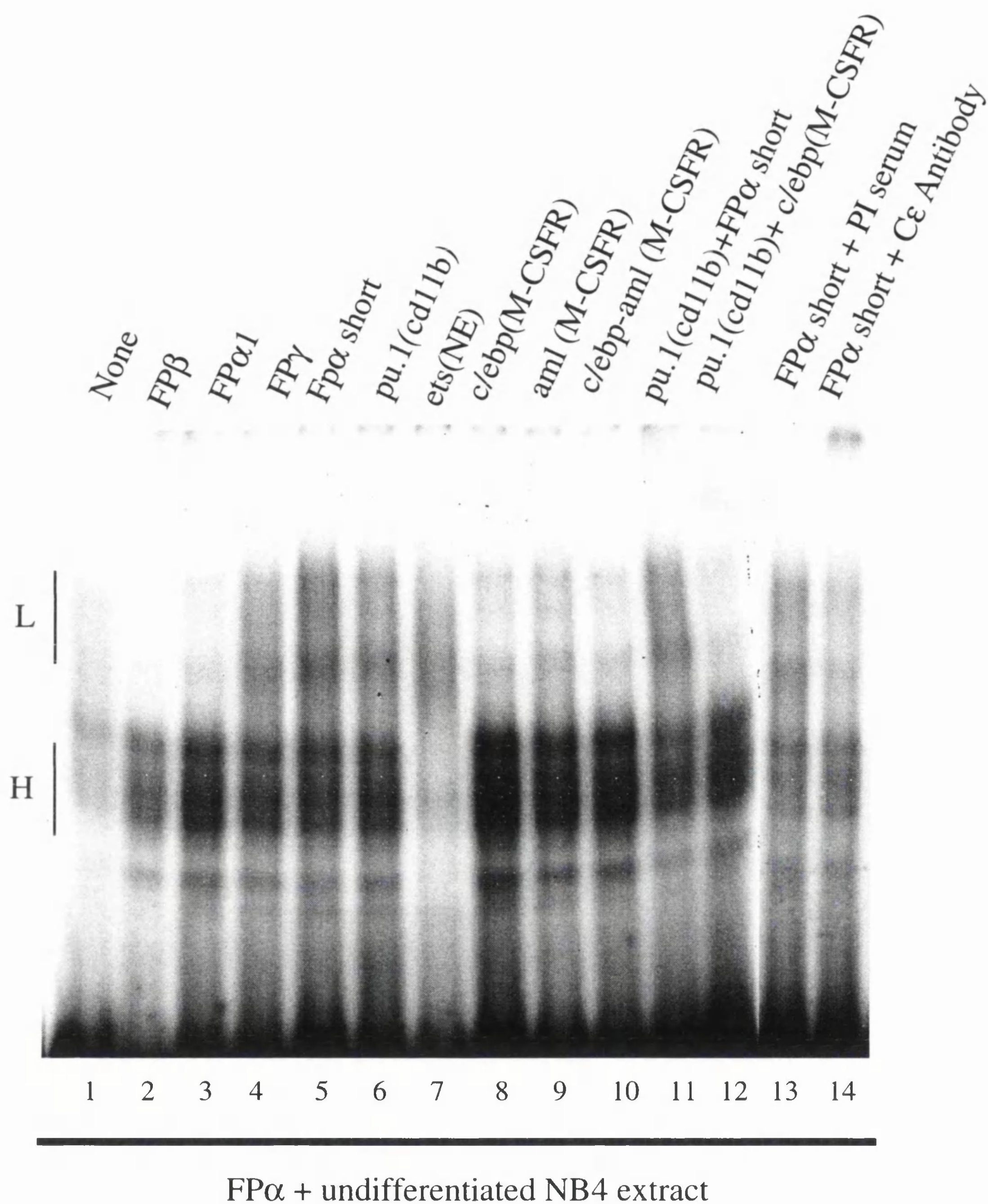


Fig.36 Enhanced binding of undifferentiated NB4 nuclear proteins to FPα following oligonucleotide competition.

5μg of undifferentiated NB4 nuclear extract, a 200-fold molar excess of unlabelled competitor oligonucleotides (shown), and [γ-^{32}p]-labelled FPα are incubated in buffer 1. Where more than one competitor is added, it is the total which is in a 200-fold molar excess with respect to the probe. Where indicated pre-immune serum (PI) and anti-C/EBPε antibody (Cε) were added to the reaction. Protein complexes formed were separated by electrophoresis through a 6% polyacrylamide gel. L and H signify Low and High mobility complexes respectively

with labelled FPα, a different pattern was observed. Whilst the increase in the intensity of the high mobility bands still occurs, this is now accompanied by a clearly enhanced binding of lower mobility complexes too (lanes 4, 5, 6 and 11). Addition of either a C/EBP-binding oligonucleotide (lane 12) or an anti-C/EBPε antibody (lane 14) reduced the formation of the Ets-binding oligonucleotide-induced complexes. In the case of anti-C/EBP antibody, a supershifted band was seen. No such reduction in band intensity or supershifted band was seen in the presence of pre-immune serum (lane 13).

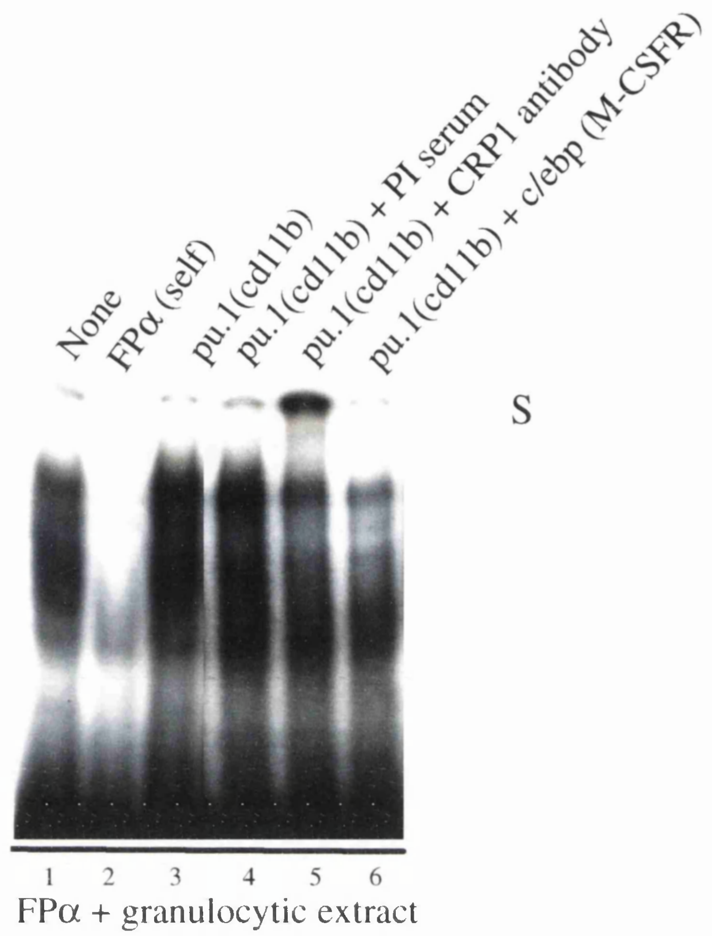
Clearly these lower mobility complexes seem to be due to enhanced binding of C/EBPε-containing factor complexes. The GABP/PU.1-binding site from the *neutrophil elastase* promoter, whilst enhancing the appearance of these (C/EBP-dependent) low mobility complexes, also results in loss of the intense high mobility complexes. A possible explanation is that these factors (which may possible be Maf family members) form a complex C/EBP in the place of Ets factors once all of these have been removed by the GABP/PU.1 binding site. Thus whilst forming a complex with C/EBPε, no free factors are left over to bind DNA alone. As well as Mafs, they may be other small leucine zipper factors such as Ig/EBP which is present rather ubiquitously (159). Competition of Ets and C/EBP factors for overlapping sites within FPα might explain why the bands seen in incubations with this probe were often quite hazy and indistinct whilst they are more clear after one of these factors has been competed away.

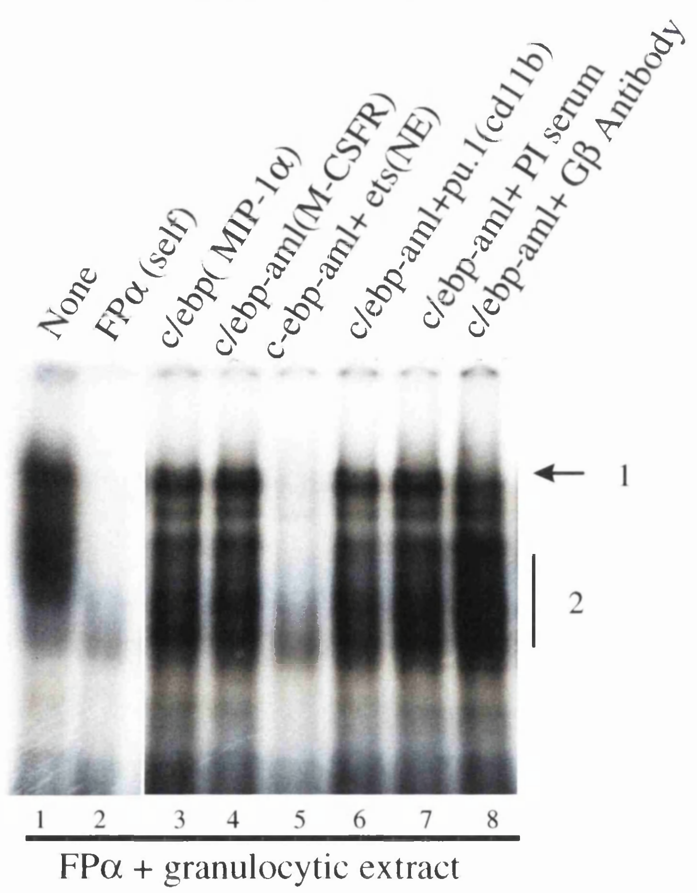
Similar studies were performed using granulocytic NB4 extract and the results of these EMSAs are shown in Fig.37. Again, co-incubation with oligonucleotides that bind PU.1, like the *cd11b* promoter site (Fig.37a), resulted in increased intensity binding. This increased binding is inhibited by C/EBP-binding oligonucleotides or by anti-C/EBPε antibodies whilst pre-immune serum has no such effect. As seen by the

Fig. 37 Enhanced binding of granulocytic NB4 nuclear proteins to FPα following oligonucleotide competition.

(a) 5μg of granulocytic NB4 nuclear protein extract was incubated with a 200-fold molar excess of competitor oligonucleotide (cd11b), together with other oligonucleotides or antisera (as indicated) in buffer 1. [γ-^{32}p]-labelled FPα is then added and complexes formed were then separated by electrophoresis through a 6% polyacrylamide gel. The band S indicates a supershifted complex formed with in the presence of the anti-C/EBPε antibody CRP1. Self (FPα) competition shows that nuclear protein binding to this probe in these conditions is specific.

(b) 5μg of granulocytic NB4 nuclear protein extract was incubated with a 200-fold molar excess of c/ebp site competitor oligonucleotides together with other oligonucleotides or antisera (as indicated) in buffer 1. [γ-^{32}p]-labelled FPα is then added and complexes formed were separated by electrophoresis through a 6% polyacrylamide gel. Arrow 1 indicates the top band of a doublet of complexes which is lost upon incubation with GABPβ antibody and the complexes which bind DNA concomitantly are marked as 2. Self (FPα) competition shows that nuclear protein binding to this probe in these conditions is specific.


a
None
FPα (self)
pu.1(cd11b)
pu.1(cd11b) + PI serum
pu.1(cd11b) + CRP1 antibody
pu.1(cd11b) + c/ebp (M-CSFR)
S
1 2 3 4 5 6
FPα + granulocytic extract


b
None
FPα (self)
c/ebp(MIP-1α)
c/ebp-aml(M-CSFR)
c-ebp-aml+ ets(NE)
c/ebp-aml+pu.1(cd11b)
c/ebp-aml+ PI serum
c/ebp-aml+ Gβ Antibody
1
2
1 2 3 4 5 6 7 8
FPα + granulocytic extract

supershifted band, the amount of C/EBPε is greater than that seen with undifferentiated extract (see Fig.36). This agrees with the results of previous studies (Fig.28). C/EBP-binding competitor oligonucleotides incubated with labelled FPα and granulocytic extract results in distinct complexes as opposed to the diffuse patterns seen with uncompeted reactions.

9.3.3 Ets-related complexes bind more strongly to FPα after C/EBP removal

The factors, whose binding is impaired by c/ebp oligonucleotide competition, are themselves competed away by the GABP/PU.1-binding ets site oligonucleotide from the *neutrophil elastase* promoter (Fig. 36b). Antibodies against GABPβ inhibited the formation of the upper band of the top doublet seen following this c/ebp site-competition (marked as 1). Pre-immune serum did not have such an effect (lane 7 as opposed to lane 8). Concomitant with the disruption of this band by the GABPβ antibody, there was an increased appearance of high mobility bands marked as 2. This pattern is reminiscent but not identical to that seen with purified GABP proteins with GABP antibodies (Fig.25b). One of the protein complexes bound more strongly in the absence of C/EBP appears to contain GABPβ and may be be GABPαβ. Removal of the GABPβ component results in enhanced binding of the high mobility factors, which may be displaced from other complexes (unbound to DNA) by released GABPα.

Chapter 10 : Western analysis - Changes in transcription factor abundance with differentiation

Changes in transcription factors abundance and expression are likely to play a role in regulating promoter activity. Increased C/EBPε expression is a recognised feature of granulocytic differentiation of myeloid cells and cell lines (374). This correlates well with the changes seen both in footprinting studies and in EMSAs. Using the available antibodies, western analysis was performed to assess changes in the abundance of other transcription factors suspected to be binding to the *defensin* promoter during granulocytic differentiation. The results of such analyses are shown in Fig.38.

One such factor which was not detected by EMSA but whose binding site is protected in DNAse1 footprinting assays is Myb. Using a monoclonal antibody derived against the C-terminal 235 amino acids of the protein, a western blot was performed using NB4 nuclear proteins prepared during a time course of granulocytic differentiation using ATRA. A significant reduction in the amount of Myb detected was seen after 48 hours of such induction (Fig38a) This correlates well with a time point just before cell division ceases in ATRA-induced NB4 cells (94).

PU.1 may be up regulated during myeloid differentiation (337) and preliminary results from EMSAs suggested that this was the case in NB4 cells. Using a monoclonal antibody derived against a GST- PU.1 fusion protein, undifferentiated and ATRA-induced (40 hours) NB4 nuclear extracts were analysed by western blotting. A clear increase in the abundance of a band migrating at the correct molecular weight of 40kDa

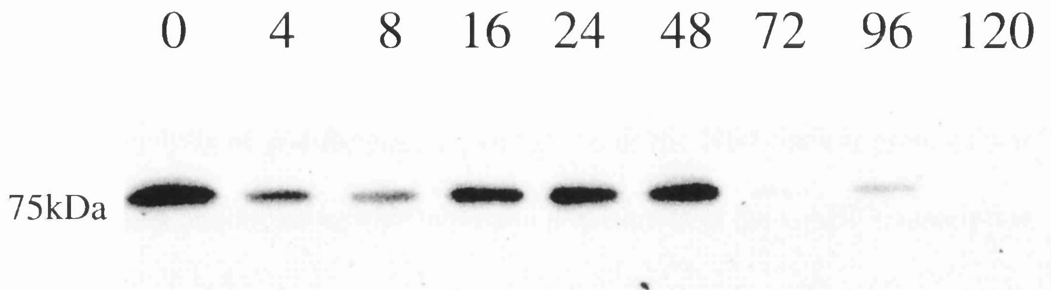
Fig.38 Western analysis of changes in transcription factor abundance with differentiation.

(a) 10μg of nuclear protein, prepared from cells at different time points into ATRA-induced granulocytic differentiation of NB4 cells (indicated in hours above each lane) was separated by electrophoresis through a denaturing SDS polyacrylamide gel. Following blotting, the gel was incubated with a monoclonal c-Myb primary antibody and protein was detected using a secondary HRP-linked antibody and chemi luminescence.

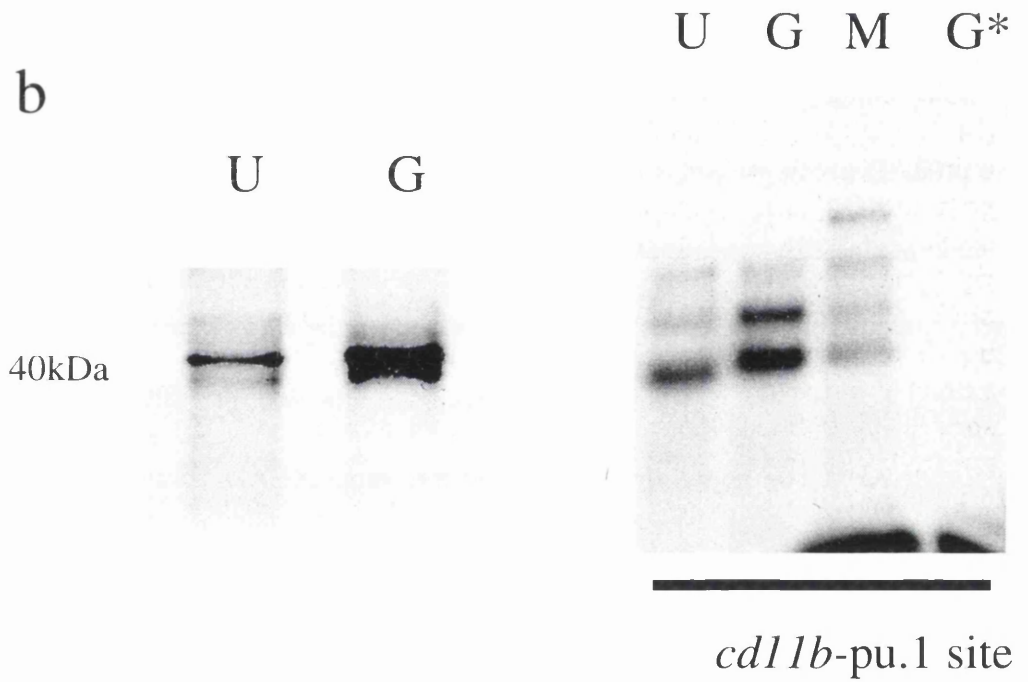
(b) The left panel shows a western blot of 10μg of nuclear protein prepared from uninduced NB4 cells (U) and cells 40 hours post initiation of ATRA-induced granulocytic differentiation (G). PU.1 is detected in the same manner as described above following incubation of the blot with a monoclonal anti-PU.1 antibody. The right panel also shows the increase in PU.1 upon NB4 differentiation by the enhanced binding to the known PU.1- binding site of the *cd11b* promoter in an EMSA. 5μg of undifferentiated (U), granulocytic (G) and monocytic (M) NB4 cell nuclear protein extract was incubated with the labelled probe in buffer 1. Binding is shown to be specific by co-incubation of a 200-fold molar excess of the same cd11b site as an unlabelled competitor (G*).

(c) 25ng of purified GABPα and GABPβ and 10μg of nuclear protein extract taken from undifferentiated NB4 cells (Nu) or differentiating NB4 cells 40 hours after initiation of ATRA-induction,(Ng) were blotted after separation by SDS-polyacrylamide gel electrophoresis. Protein was then detected by incubation with anti-GABPα or GABPβ antibodies and then by chemiluminescent detection as described.

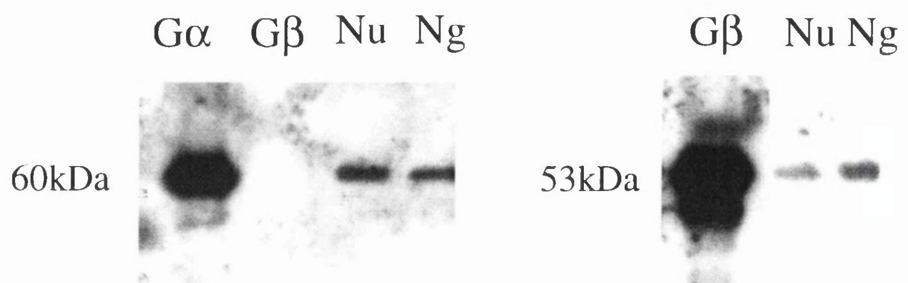
a



b



c



was detected (Fig.38b). An EMSA is also shown using the recognised PU.1 binding site of the *cd11b* promoter, which also shows a clear increase in the characteristic PU.1 doublet with granulocytic extract with respect to undifferentiated or even monocytic extract (24-hour induction).

Western analysis of undifferentiated and granulocytic NB4 nuclear proteins was also performed using antibodies against the α and β sub-units of the GABP transcription factor. EMSA results had given some indication that the GABPα sub-unit but not the GABPβ sub-unit was detectable in undifferentiated extract. This was contrary to what was indicated in previous publications, (315) that both GABP sub-units were ubiquitously expressed in most tissues. Western analysis indicated that both sub-units were in fact efficiently expressed in both undifferentiated and granulocytically differentiating NB4 cells. Neither were large changes in molecular weight of GABPα or β, as a result of post-translational modifications, apparent between undifferentiated and differentiating cell extracts. This suggests that any modifications may be happening by re-distribution of modifier groups rather than de novo addition.

Importantly whilst GABPβ may not be bound to its target site DNA through GABPα in undifferentiated NB4 cells, it is definitely present. Having knowledge of the main transcription factor binding sites within the *defensin* promoter and evidence of transcription factors bound to these sites, functional testing was performed to assess the importance of these various factors *in vivo*.

Chapter 11 : Characterisation of the reporter gene system for transfection studies

The main interest of our study was to study the function of the *defensin* promoter *in vivo*, to intervene experimentally and assess the results of such interventions. In order to do so, a good reporter gene system that can function effectively within the test environment is essential. Other members in our laboratory had through their own studies, noted, that, when analysing promoter activity within myeloid cells (HL60 myeloblastic leukaemia cells), reporter molecules which were exported from the cell proved greatly advantageous over other more commonly used reporters. Luciferase reporter activity was found to be weak in undifferentiated HL60 cells and was completely lost once the cells were induced to differentiate (380). Assaying luciferase reporter activity, like chloramphenicol acetyl transferase (CAT) reporter activity, requires lysis of the transfected cells and assaying the activity within this lysate. Myeloid cells are the organism's host defence system against intruding pathogens and as such are laden with nucleases and proteases far beyond most other cells. One explanation for these results was therefore that the enzyme was destroyed by the said proteases upon cell lysis (despite the use of various protease inhibitors) and was therefore an ineffective method to study promoters in these cells. For this reason, experiments were performed using a human growth hormone gene driven by the test promoter as a reporter whilst placental (secreted) alkaline phosphatase expressed from a cDNA driven by an early SV40 promoter(362) was used as a transfection control. Since both reporter and transfection control molecules were secreted by the cells, testing

involved a simple assay on an aliquot of the medium in which the cells were growing. This was the system I had then determined to use for the study of *defensin* promoter activity in the myeloid NB4 cells.

11.1 Anomalous up-regulation of empty growth hormone reporter vector

Initial studies presented a serious problem that required amending before any experiments could be performed. Co-transfection of expression vectors producing my test transcription factors together with the growth hormone reporter gene driven by a *defensin* promoter fragment caused a good increase in the reporter gene activity. This was unfortunately invalidated by a similar increase in the reporter activity obtained upon co-transfection of an expression construct together with the empty reporter vector (the growth hormone gene not driven by any promoter). Such an effect was seen with two different co-transfected transcription factors (Fig.39). This initial problem with the reporter system was compounded by similar problems of up-regulation of the empty reporter vector activity by the process of granulocytic differentiation itself (see Fig.41a). Since the change in promoter activity with differentiation was my main area of interest, steps were taken to correct this reporter system in order for valid experiments to be carried out.

11.2 Modification of the growth hormone reporter vector

The growth hormone reporter construct I had used was described by Selden et al (381). Analysis of this construct led me to identify a number of potential

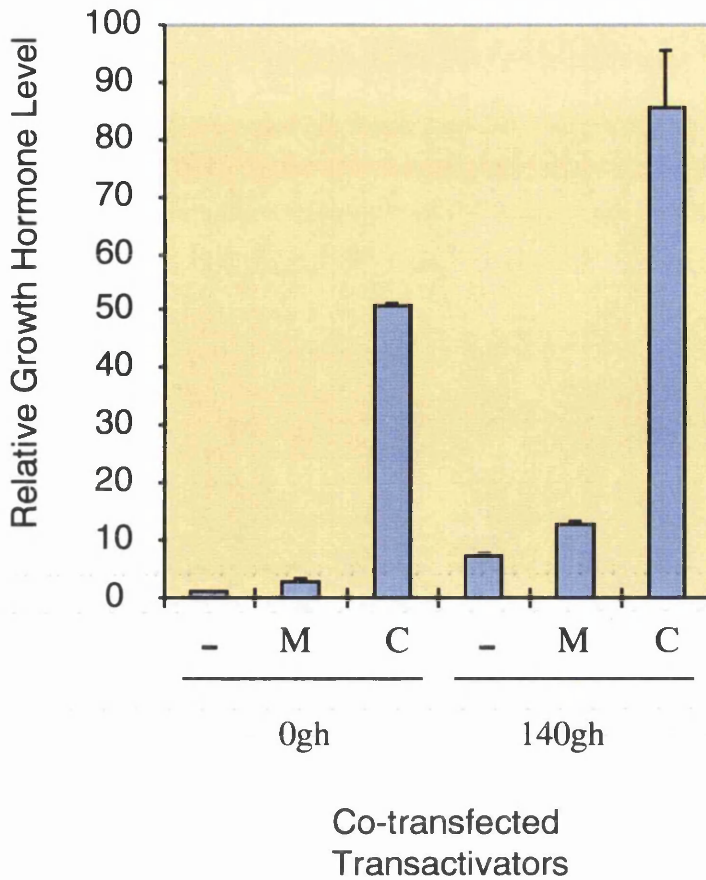


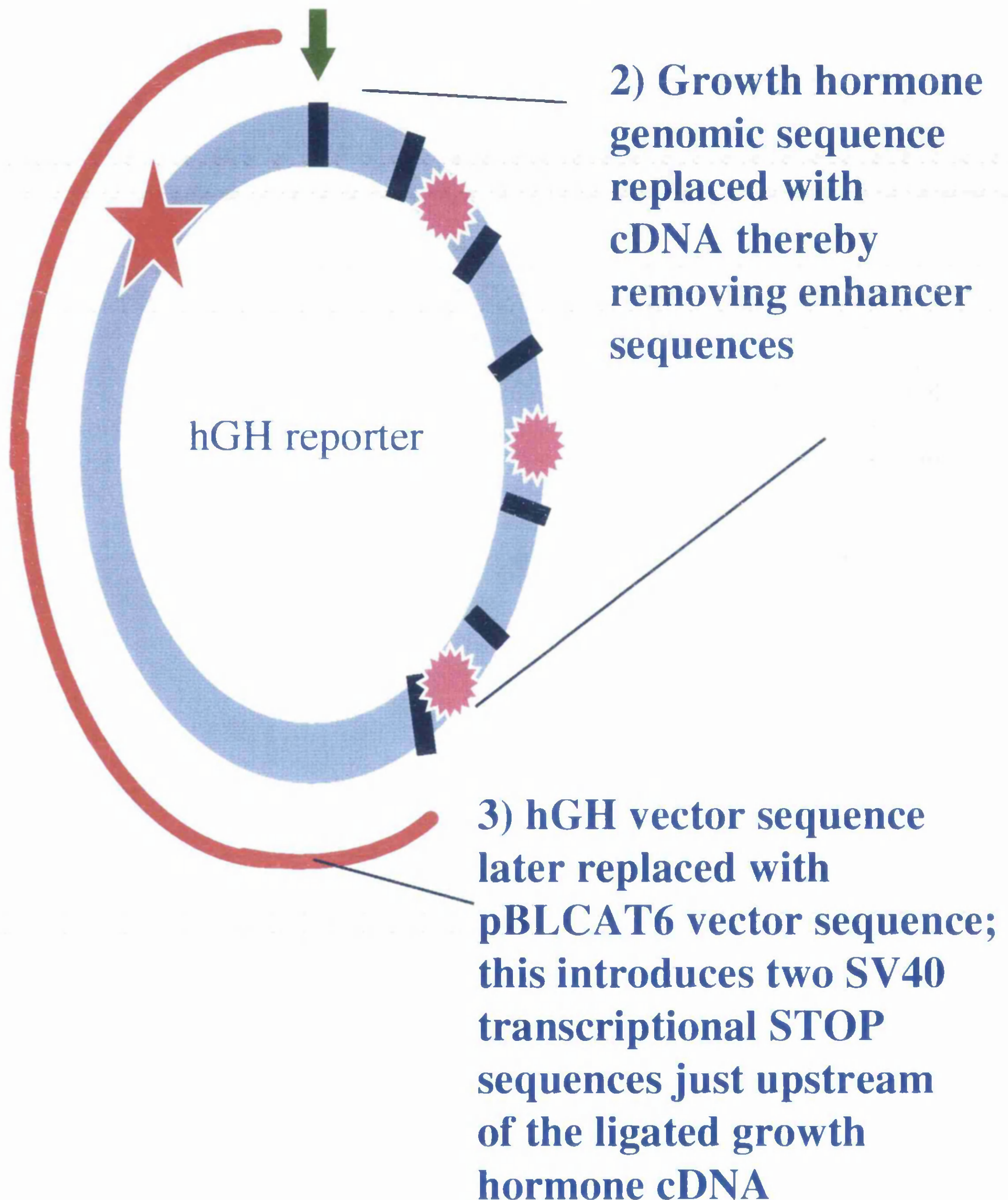
Fig.39 Transactivation of the empty genomic growth hormone reporter vector.

Average growth hormone levels, are indicated, relative to that measured after transfection of the reporter vector alone (taken as 1). All results represented are means of at least 4 separate experiments. M indicates 5μg of co-transfected c-Myb expressing vector whilst C signifies 5μg of co-transfected C/EBPα expression vector. It is evident that the empty reporter vector (0gh) is up regulated in a manner similar to the defensin promoter fragment-bearing vector (140gh).

C/EBP-binding sites within the intron sequences of the growth hormone gene. Since C/EBP co-transfection had caused a large up-regulation of the empty reporter vector, removal of these sequences would, theoretically, help reduce this vector-dependent reporter activity. Whilst these intron sequences may be acting as enhancer sequences, no transcription should have been possible in the absence of a promoter, suggesting that a spurious promoter sequence was present within the backbone vector sequence derived from the pUC12 plasmid. In order to remedy these two points, I cloned a human growth hormone cDNA (a kind gift from Paul Robbins) in place of the genomic growth hormone sequence. This I modified by introducing a consensus Kozak sequence just upstream and an efficient polyadenylation signal downstream of the cDNA. I also introduced two synthetic transcriptional stop and polyadenylation signals upstream of the multiple cloning site such that any mRNA initiating upstream of these sequences would be terminated and would not extend into the reporter gene sequence. These changes are marked as points 1 and 2 in the schematic in Fig.40. This reporter gene was much improved with respect to vector dependent activity caused by granulocytic differentiation (despite a now anomalous up-regulation upon monocytic differentiation). However, there was still considerable up-regulation of the promoter by C/EBP expressing vectors (See Fig. 41b). Further changes were therefore required.

A similar problem with spurious vector-dependent activity had been previously detected and corrected in a CAT reporter construct pBLCAT3 by Boshart et al(360). In this case, sequences upstream of the multiple cloning site where promoters were introduced for study, had been noted to have promoter activity, and the deletion of these sequences together with insertion of two SV40 derived transcriptional stop sequences, markedly reduced vector sequence dependent reporter activity. In order to produce the best reporter gene for my studies, the modified human growth hormone cDNA from my

Fig.40 Corrective measures applied to the genomic growth hormone reporter vector. Black rectangles along part of the vector, schematically represent intron-exon boundaries within the growth hormone gene, with pink starbursts showing potential enhancer sequences within the introns. The red star signifies an anomalous promoter upstream of the multiple cloning site of the reporter vector (indicated by the green arrow). Steps take to correct the reporter gene are noted numerically with modifications 1 and 2 having been incorporated first and change number 3 being introduced into the final reporter gene vector.


1) Synthetic AATAAA
(inserted upstream of the
multiple cloning site in
the original hGH vector)
2) Growth hormone
genomic sequence
replaced with
cDNA thereby
removing enhancer
sequences
hGH reporter
3) hGH vector sequence
later replaced with
pBLCAT6 vector sequence;
this introduces two SV40
transcriptional STOP
sequences just upstream
of the ligated growth
hormone cDNA

improved reporter vector was cloned into the corrected backbone of the improved pBLCAT6 reporter vector in place of the CAT cDNA. These changes are marked as point 3 in the cartoon in Fig.40.

11.3 Differential responses with new and old hGH vectors

11.3.1 Different vector responses to differentiation and co-expressed activators

Fig. 41 shows the improvements to the new hybrid PBLCAT6/hGH vector as opposed to the initial corrected vector and the original genomic growth hormone vector. Fig. 41a clearly shows a marked reduction in granulocyte differentiation-induced activity. The unexpected increase in monocytic differentiation-induced up-regulation in the activity of the initial corrected vector is also shown. The final reporter vector, though retaining minimal monocytic induced activity, is much improved when used in both lineages.

The modifications to the reporter vector reduced spurious vector sequence-dependent promoter activity even in the case of co-transfections with expression vectors, as can be seen in Fig.41b. The three lanes marked for each vector 1, 2 and 3 show the effects of 50ng, 500ng and 5μg of co-transfected C/EBPα-expressing vector respectively.

11.3.2 Different activity of promoter fragments in old and new hGH vectors

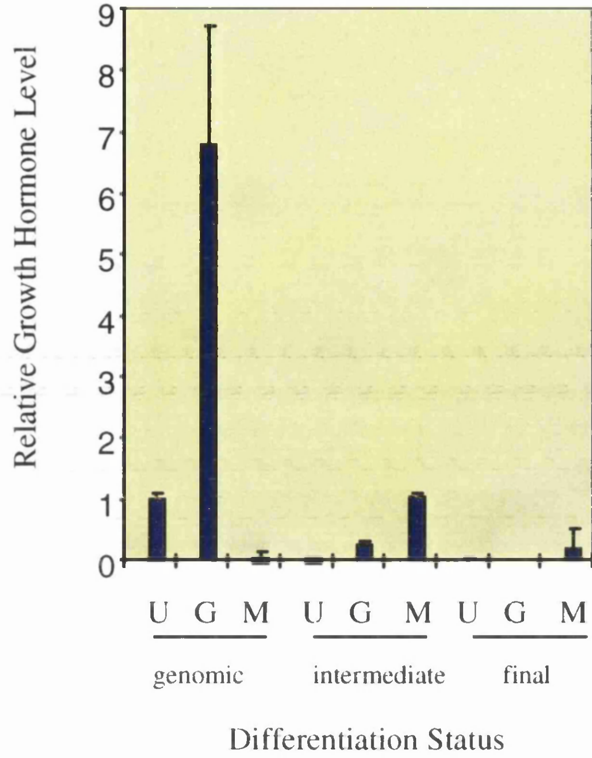
Since a number of experiments had been carried out previously in our laboratory using HL60 cells, I decided to compare the activity of *defensin* promoter fragments

Fig.41 Up regulation of old and new, growth hormone reporter vectors, by differentiation and transactivators.

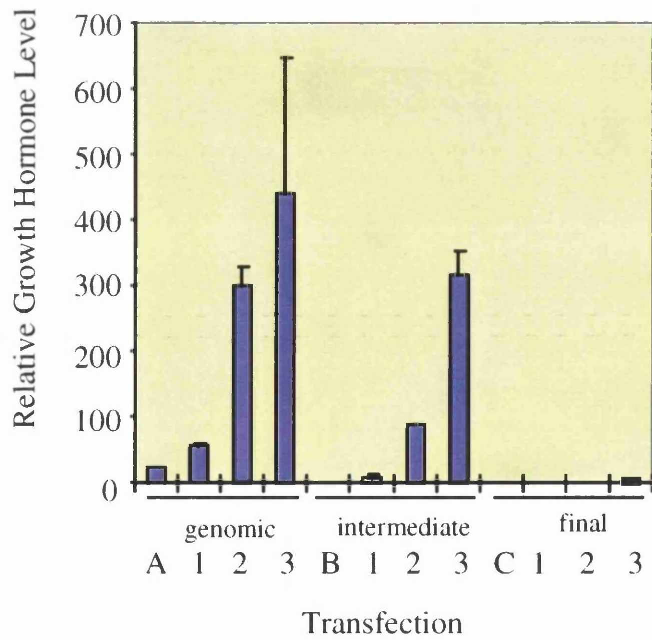
(a) From left to right are indicated the relative growth hormone levels measured after transfection of the genomic growth hormone reporter, the intermediate modified reporter and the final reporter vectors into undifferentiated (U), granulocytic (G) and monocytic (M) NB4 cells respectively. Growth hormone levels are quantified relative to that measured following transfection of the genomic vector into undifferentiated cells.

(b) Relative growth hormone levels measured following transfection of the genomic (A), intermediate (B) and final (C) growth hormone reporter vectors into undifferentiated NB4 cells with 50ng (1), 500ng (2) or 5μg (3) of C/EBPα-expressing vector. Values are quantified relative to the level of growth hormone measured following transfection of the final vector (C) without any co-transfected activators (taken as 1).

a



b



driving the new reporter vector with the activity obtained by the same fragments driving the original genomic growth hormone vector. The results of these experiments are shown in Fig.42. The results obtained with the old vector repeated the pattern seen with earlier HL60 experiments (Fig.42a). Those with the new vector showed two interesting and important changes (Fig.42b). Firstly it is apparent that the absolute amount of growth hormone activity produced by this new vector is considerably less than that produced by the original vector (not shown) which means it may not be optimal for use in studying cells which are transfected as poorly as HL60. This reduced activity may be due to the lack of enhancers within the intron sequences and the loss of one promoter in the vector sequence.

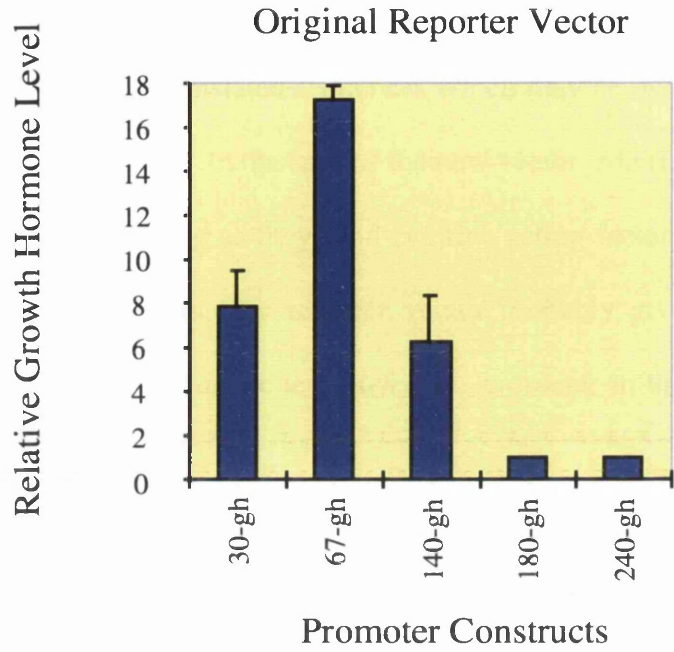
The other difference is the change in the pattern of reporter activity as expressed by the different *defensin* promoter fragments. In both cases, all values are expressed as relative to the activity of the -240/+15 promoter, (which is taken arbitrarily as 1). As can be seen, fragments cloned into the old vector present a pattern where the bulk of the promoter activity is present in very short *defensin* upstream sequences and peak activity was obtained from a -67/+15 sequence. Greater lengths of upstream sequence resulted in reduced activity. When assessing the activity of the same promoter fragments in the new vector, the shortest fragment -30/+15 was one of the least active. Promoter activity increased gradually to reach a peak activity in the -140/+15 fragment that had a similar activity to that obtained from a -240/+15 promoter fragment-driven construct. The -180/+15 fragment is relatively inactive- a feature that was later also found in NB4 cells. Thus the overall pattern is one where with the new vector, adding in other site within this sequence enhances activity whilst with the original vector, adding in more promoter sequence reduces the activity. One possible explanation for this is that when using the older reporter system, much of the growth hormone expression is dependent on the

Fig.42 Different reporter constructs show different patters of deletion mutant promoter activity.

(a) Relative growth hormone activity following transfection of the genomic growth hormone reporter gene driven by different defensin promoter fragments (as indicated) into HL60 myeloblast cells.

(b) Relative growth hormone values following transfection of the final growth hormone reporter vector into HL60 cells driven by the same promoter deletion fragments as in (a). In both cases, growth hormone levels are quantified relative to the value measured following transfection of the –240/+15 deletion mutant construct.

a



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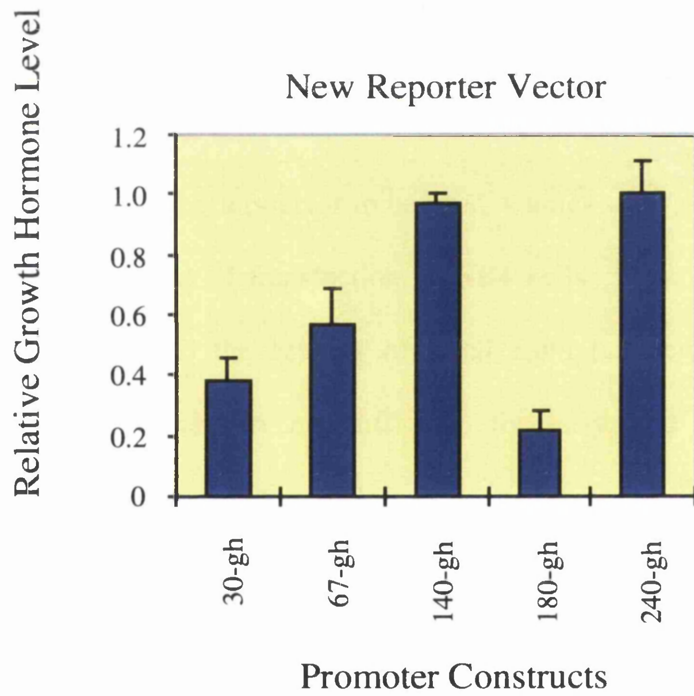


spurious promoter sequence in the vector and not the test promoter. Therefore, inclusion of longer test promoter sequences actually distance this promoter (and its associated transcriptional start site) from the reporter gene itself such that mRNA produced by this promoter end up having elongated untranslated sequences which may be deleterious to eventual translation of the reporter gene. In the case of the new vector, addition of more transcription factor binding sites, so long as they bind positive acting factors, result in increased activity. This shows that this new reporter vector probably gives a more accurate assessment of the real activity of the test (*defensin*) promoter in this myeloid cell system.

11.4 Optimising the new hGH reporter test system

11.4.1 Optimisation of transfection conditions

Having decided upon the test reporter vector to be used, studies were performed in order to establish optimal conditions of transfection in NB4 cells. These cells are easier to transfect than HL60 cells so the activity obtained from the new growth hormone reporter construct was likely to be sufficient for easy and accurate measurement yet optimisation was important nonetheless.

A standard amount (5μg) of control plasmid which expresses placental (secreted) alkaline phosphatase, was transfected into NB4 cells (as described in materials and methods) using a range of different voltage settings. Alkaline phosphatase activity is measured in the medium collected after overnight recovery of the cells post-transfection. These experiments suggested that optimal transfection occurs with a voltage of 180 to 200 V. The latter voltage was chosen for all subsequent

experiments. The total amount of DNA used in transfections is known to influence the efficiency of transfection (382). Therefore, using the optimised transfection voltage a number of transfections were carried out with varying amounts of total DNA (made up by adding carrier pBluescript DNA over and above the 5µg of alkaline phosphatase construct). 30µg of total DNA resulted in the optimum reporter activity. Using these two optimised features, transfections were carried out and medium was collected at different time points following transfection to assess when reporter activity is first detected and when it is best to assay the medium. Alkaline phosphatase activity above background levels was first detected at about 6 hours. A significant amount of activity (about 50% of that seen after 20 hours) was detected at 8 hours post transfection and also at later times well after this point (Fig. 43).

11.4.2 Control studies with uninduced, ATRA-exposed and differentiated cells

Once the optimal conditions for transfection had been established using the alkaline phosphatase plasmid, the actual reporter gene construct was tested. It is important to quantify the production rate of the reporter molecule such that medium can be assayed before plateau is reached. This will allow differences in the amount of growth hormone quantified, to be more accurately related to the activity of the test promoter. It is also important to perform similar tests during transfection into differentiating cells so as to ascertain that results obtained from these studies are similarly relevant. Using a test *defensin* promoter construct (-240/+15), and the conditions optimised using the alkaline phosphatase promoter, transfections were carried out and medium removed for assaying growth hormone concentration at different time points following the transfection. As can be seen in Fig.44, growth

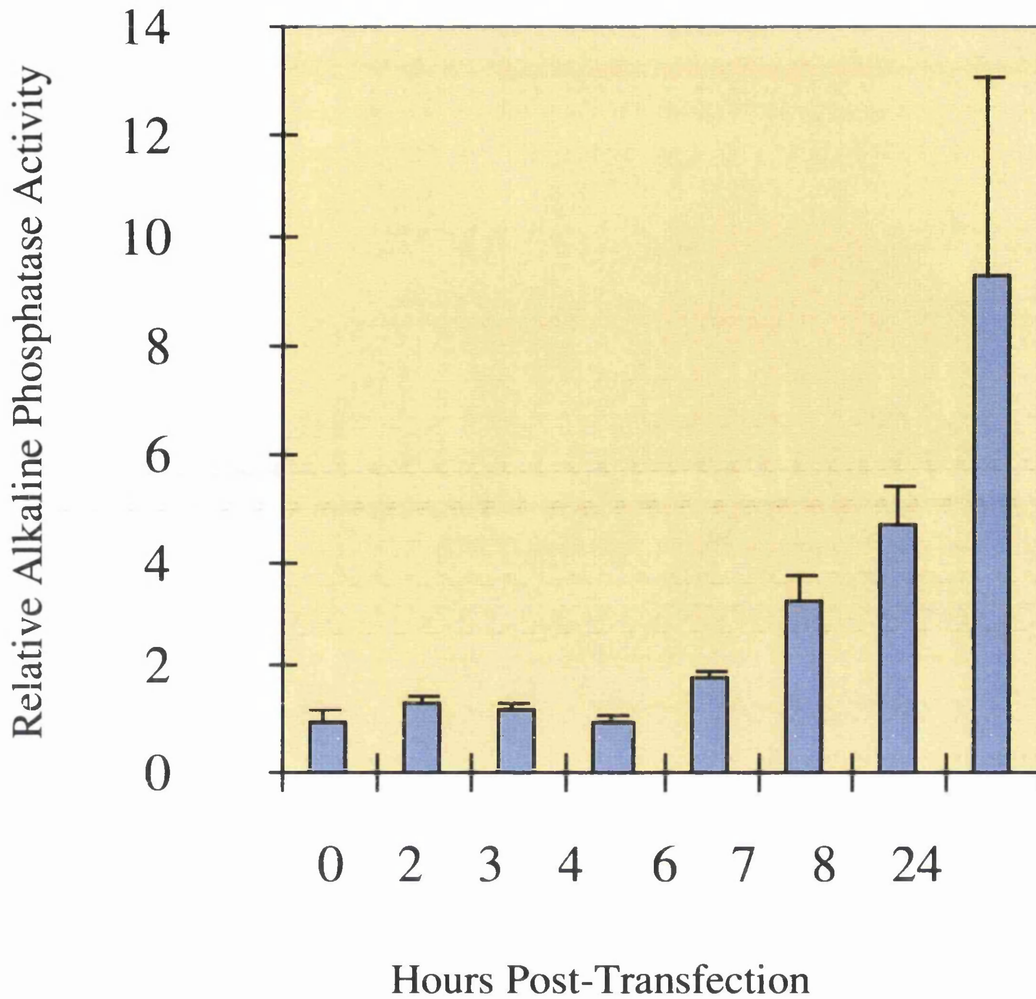


Fig.43 Identifying the best time to withdraw medium for testing reporter activity. Following transfection of the alkaline phosphatase reporter construct,into undifferentiated NB4 cells, samples of medium were removed from the flasks containing the transfected cells at different time points. Enzyme activity is measured, average values from four different transfections are calculated and the mean level of activity relative to that one hour post transfection is plotted against the time elapsed since transfection.

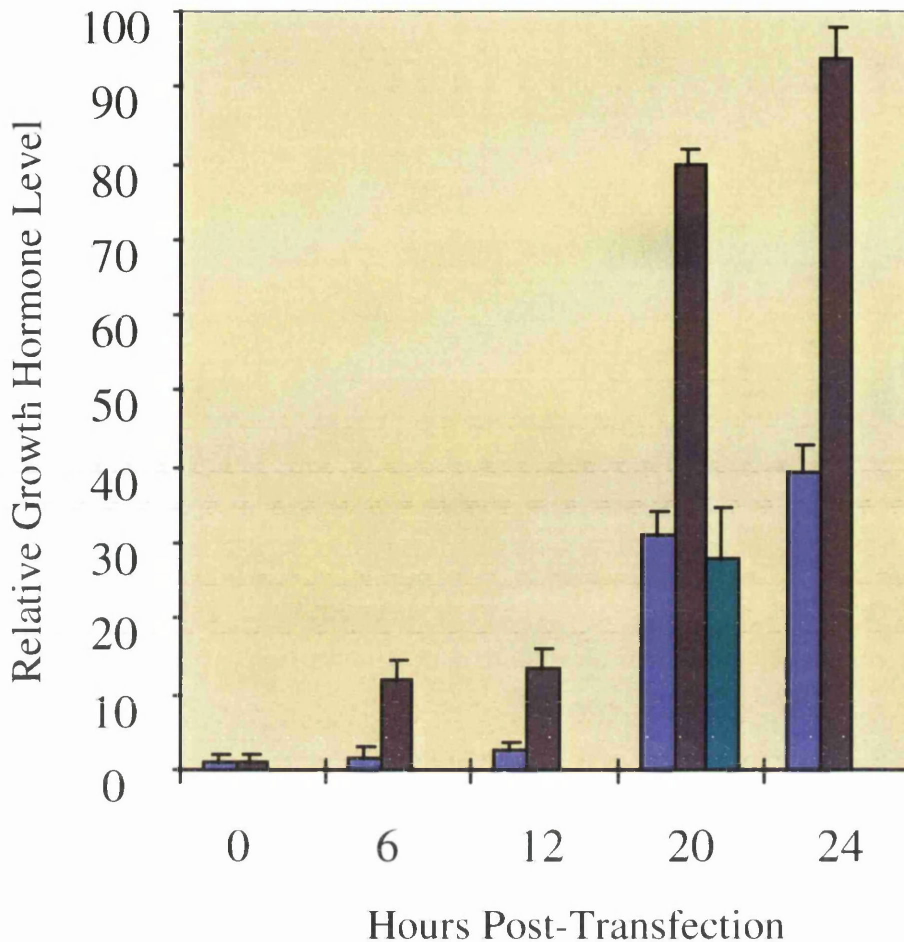


Fig. 44 Assessing growth hormone production with differentiation.

At different time points following transfection of undifferentiated NB4 cells, medium is removed and growth hormone measured. An average is calculated from four independent transfections and presented relative to the growth hormone level measured in just transfected cells (taken as 1). These values are indicated as the blue bars. Growth hormone levels are similarly measured following transfection into NB4 cells differentiated by ATRA for 20 hours These average values, (standardised to the level of transfected undifferentiated cells) are indicated by the brown columns. The green column indicates the average growth hormone level measured 20 hours after transfection of undifferentiated NB4 cells exposed to ATRA for 10 hours.

hormone in concentrations above background was detected as early as six hours after transfection in the ATRA-induced cells (brown columns) but was barely detectable up until 12 hours in the uninduced cells (blue columns). In both cases, a major increase in the reporter activity (of similar rate) was seen between 12 and 20 hours post-transfection but even at this time point the expression of growth hormone had not yet reached a plateau but was still increasing. Therefore I decided to assay medium taken 18 to 20 hours post-transfection since the growth hormone level measured at this time point should give a fair indication of the activity of the test promoter.

One very important test was to ensure that ATRA had no direct effect on *defensin* promoter activity (due to ligand-bound RARα -induced transactivation) which could be misconstrued to be a differentiation-induced effect.

In order to assess this, ATRA was added to undifferentiated NB4 cells 10 hours after transfection of a *defensin* test promoter construct but 8-10 hours prior to assaying the medium for growth hormone. As can be seen in Fig. 12, the increase in *defensin* mRNA during ATRA-induced differentiation of NB4 cells is first detected at 16 hours and is not present at 8 hours following initiation induction. Thus if ATRA directly enhances the promoter activity it should be detected in this experiment whilst there is not enough time to cause an increase in the promoter activity secondary to differentiation of the transfected NB4 cells.

As can be seen, this test excludes a direct effect of ATRA in up-regulation of the *defensin* promoter in this test system (green column 20 hours). This correlates with studies in HL60 cells where the kinetics of ATRA-induced *defensin* mRNA up-regulation excluded a direct effect (52). The lack of consensus retinoic acid receptor recognition elements (RAREs) within the *defensin* promoter also concurs with the lack of direct RARα- dependent transactivation.

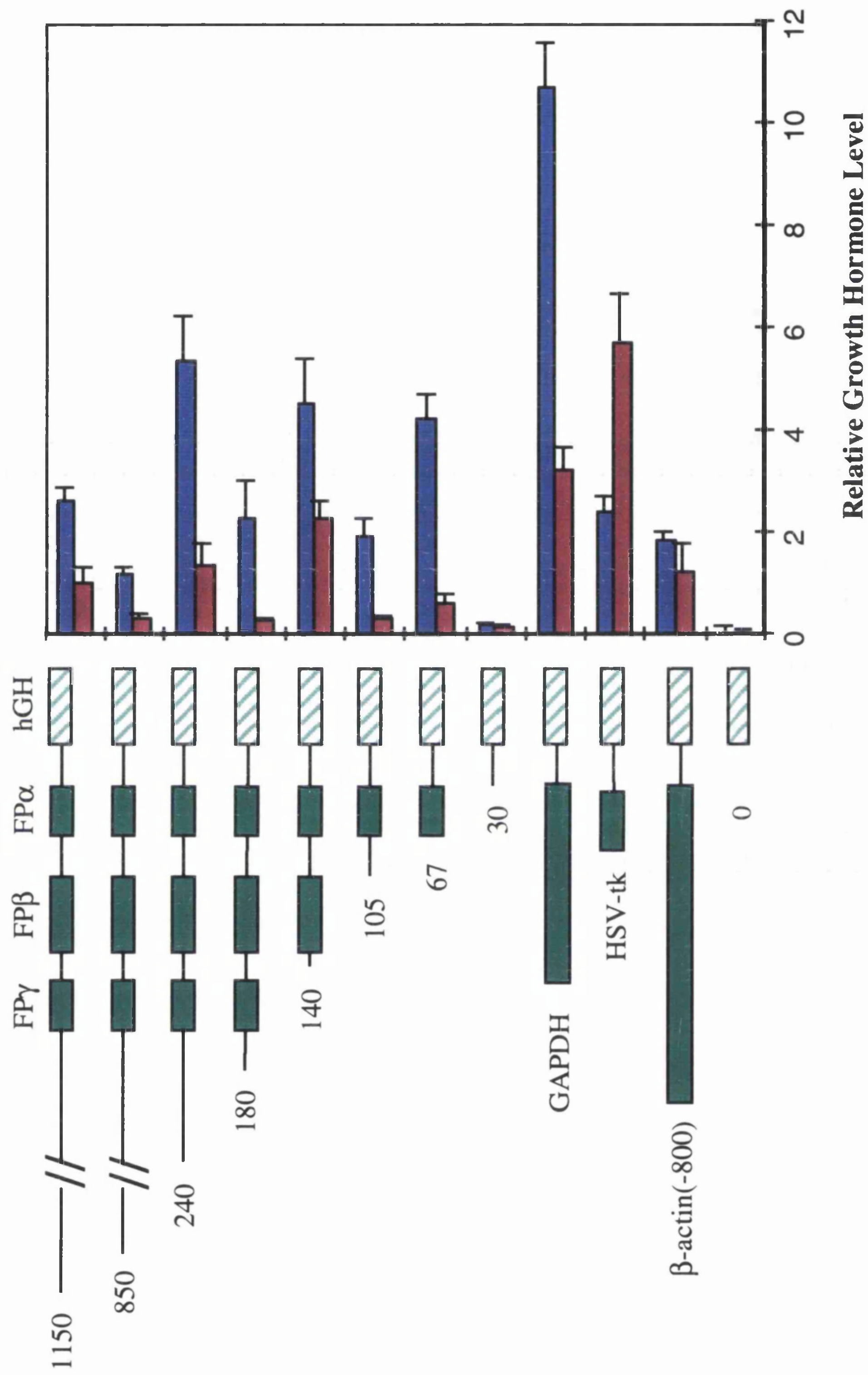
Chapter 12 : Transfection studies in NB4 cells

12.1 Up-regulation of *defensin* promoter activity upon differentiation

Various lengths of the sequence 5' of the *HNP-1* gene, each extending 3' to nt +15 relative to the primary transcription start site were cloned into the new growth hormone reporter vector resulting in a number of test promoter constructs. Many of, but not all the promoter fragments had been generated previously (380). The test constructs are schematically represented in Fig. 45 on the left side of the figure. These different *defensin* promoter deletion mutant constructs were transfected into ATRA-induced and uninduced NB4 cells together with a number of different controls and medium was removed for growth hormone assay (as described in the materials and methods). The bars on the right of the figure indicate relative growth hormone activity in undifferentiated (red) and differentiated (blue) NB4 cells.

The different effects on the different control promoters show that differentiation is not causing a non-specific up-regulatory effect. The β-actin promoter-driven reporter gene is not up-regulated to any great extent (1.5 fold), a result which correlates well with the picture seen in the northern analysis of β-actin mRNA (Fig.13). The minimal up-regulation may be a non-specific effect of differentiation on the construct. The herpes simplex virus thymidine kinase (HSVtk) promoter is down regulated by differentiation, an effect that may reflect the increased expression of repression factor Evi-1 upon myeloid differentiation (383). Evi-1 has been shown to down-regulate HSVtk promoter activity (384). The Glyceraldehyde phosphate dehydrogenase (GAPDH) promoter is strongly up regulated on the other hand. C/EBP factors, which

Fig. 45 Functional activity of *defensin* promoter constructs in undifferentiated and differentiated NB4 cells. Cells were transfected with 5μg of each deletion mutant construct (shown schematically on the left of the figure) together with 20μg of pBluescript as carrier DNA and 5μg of pSV2Apap as internal control. Mean hGH levels calculated from measurements taken of 4 separate transfections into undifferentiated cells. These are normalised to alkaline phosphatase activity and expressed relative to the mean normalised activity of the longest (-1150/+15) promoter construct (taken as 1). These are shown as red bars.
To assess the increase in promoter activity upon differentiation, the alkaline phosphatase internal control activity is disregarded since changes to the activity of the SV40 promoter which drives its expression with differentiation cannot be gauged. The relative up-regulation of the *defensin* promoter construct is calculated as the ratio of the average hGH level measured after transfection into differentiated cells to the average level measured in undifferentiated cells. This value is then multiplied by the undifferentiated normalised value for that construct, and the value shown as the blue bars. Mean hGH levels are calculated from measurements from four separate transfections. Growth hormone reporter constructs driven by other promoters ; glyceraldehyde phosphate dehydrogenase (GAPDH), herpes simplex virus thymidine kinase (HSV-tk) and β-actin, indicate that the effects of differentiation of different promoters is specific to the particular promoter.



FPγ
FPβ
FPα
hGH
1150
850
240
180
140
105
67
30
GAPDH
HSV-tk
β-actin(-800)
0
0
2
4
6
8
10
12
Relative Growth Hormone Level

are up regulated during myeloid differentiation, may be responsible (385). The overall pattern of deletion mutant promoter activity seen in undifferentiated NB4 cells is not dissimilar to that seen in HL60 cells with the new reporter construct (see Fig.42b).

Minimal promoter activity was detected within the sequence -67/+15, which incorporates FPα. This is significantly shorter than the minimal promoter (-83/+82 with respect to the transcription start site) identified in *defensin*-expressing HL60 cells by Ma et al (54). The promoter sequence appears to be tissue-specific since it has negligible activity in endothelial (HeLa) cells (see Fig.49). The inclusion of further upstream sequences (-105/-67) slightly reduced activity whilst the fragment -140/-105 (FPβ) increased it considerably and the -180/-140 (FPγ) sequence again reduced it. Inclusion of more 5' sequences had variable effects but with these longer fragments which we did not study in detail as to protein binding, non-specific effects may predominate. Therefore, the -140/+15 fragment is the shortest length to show strong promoter activity in undifferentiated cells.

To determine the effect of differentiation on the activity of the *defensin* gene promoter, NB4 cells were induced with retinoic acid and, 20 hours later, transfected with promoter-reporter gene constructs, the alkaline phosphatase construct and carrier DNA. A concurrently grown batch of uninduced cells was simultaneously transfected with the same mixture. Medium was collected for reporter assay 20 hours after transfection.

While there was only a 2.5-fold increase in the activity of the longest (-1150/+15) promoter construct, a much more marked increase (7-fold) occurred with shorter constructs that included FPα (-67/+15 and -105/+15). In contrast, the -140/+15 construct (which also includes FPβ) showed only 2-fold greater activity in differentiated cells, though, as noted earlier, this construct had relative high activity in undifferentiated

undifferentiated cells. Interestingly, the activity of the construct incorporating FP α,β and γ (-180/+15) which was low in undifferentiated cells showed a marked 8-fold increase after differentiation. It is notable that the -240/+15, -140/+15 and -67/+15 promoter constructs, which all contain FPα, had the same elevated level of activity after differentiation despite displaying considerably different activities in undifferentiated cells. On the other hand, the greatest increases in promoter activity were seen with those constructs with the lowest activities (-180/+15 and -105/+15) prior to induction of differentiation. The shortest construct (-30/+15), which lacked FPα, had negligible activity even in differentiated cells.

The high activity of the minimal promoter construct (-67/+15) upon differentiation suggests that factor binding upstream of nt -67 is not essential for expression in differentiating cells. The greater activity of the -140/+15 promoter sequence in undifferentiated cells, suggest that factors (possibly c-Myb) bind to the -140/-67 fragment which co-operate with the factors bound to the minimal promoter (-67/+15) to enhance activity. In differentiated NB4 cells, unlike in undifferentiated ones, factors bound to the -67/+15 construct resulted in maximum promoter activity. GABP, (Fig.25a), AML-1 (53) and PU.1 (54) have all been shown to bind within this length of *defensin* promoter sequence. All three of these factors are known to co-operate with C/EBPs (which also appear to bind FPα) on different promoters (185;218;238;321). GABP also co-operates with AML-1(349). Increased abundance of transcription factors that can bind to the minimal promoter sequence with differentiation, (e.g. AML-1(338) C/EBP(386)) may provide alternative synergistic partners to the factors binding there in undifferentiated cells. This obviates the need for factors binding further upstream (such as at FPβ).

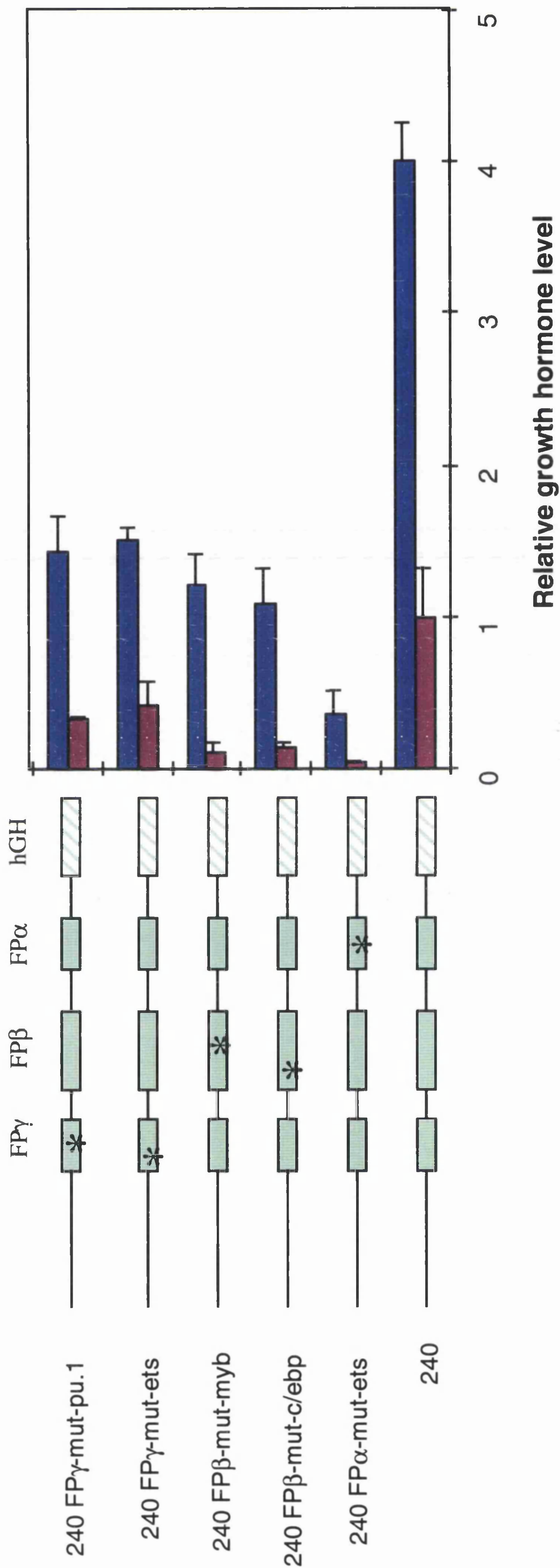
Inclusion of promoter sequences -180/-140 and -105/-67, respectively, strongly inhibited the activity of shorter promoter constructs, and the inhibitory effect was not relieved by differentiation. One possible cause of this is the presence of inhibitor binding sites. A potential site for the inhibitory protein E4BP4 (387) was found at position -97/-85 superimposed on a c/ebp site, and a second one, for the δEF1 repressor protein (388), at -180/-140. It has been shown that a human truncated variant of δEF1, ZEB, is capable of repressing an early myeloid gene in mice and that this repression is relieved by a combination of Ets and Myb, transcription factors (212).

Inclusion of further upstream sequences overcomes this repression and restores good promoter activity. This suggests that it is the ratio of inhibitors to activators bound to the DNA and their co-operative and/or inhibitory interactions, which is responsible for the relative activity of each individual construct.

12.2 Mutation of C/EBP-, Myb- and Ets-binding sites reduce *promoter* activity

In order to study further the relative importance of the protein-binding sequences FP α,β and γ in undifferentiated and differentiated cells, a number of site-specific mutations were introduced into the -240/+15 promoter construct. These mutations were: a mutated ets site in FPα; mutated c/ebp or myb sites in FPβ and mutated ets or pu.1 sites in FPγ. (The specific mutated sequences are all indicated in the materials and methods section 5.3.9.1). Whilst all the mutations resulted in reduced promoter activity in undifferentiated and differentiated cells (Fig. 46), the extent of this inhibition varied. The increase in promoter activity following induction of differentiation also differed

Fig. 46. Effects of mutating transcription factor binding sites on promoter activity. The -240/+15 *defensin* promoter constructs (5μg) was transfected into undifferentiated and granulocytic NB4 cells, as was 5 μg of each mutated construct together with carrier DNA and internal control as described in Fig.45. The mutation is represented in each construct on the left side of the figure as an asterisk within the mutated footprint. In undifferentiated cells, the mean hGH level in each case was normalised against alkaline phosphatase activity and then expressed relative to the value for the unmutated -240/+15 construct. The up-regulation of promoter construct activity with differentiation is calculated as described in Fig.45. Red bars; undifferentiated cells, blue bars; differentiated cells. Mean hGH levels are calculated from measurements from four separate transfections.



240 FPγ-mut-pu.1
240 FPγ-mut-ets
240 FPβ-mut-myb
240 FPβ-mut-c/ebp
240 FPα-mut-ets
240
FPγ
FPβ
FPα
hGH
0
1
2
3
4
5
Relative growth hormone level

with each individual mutant. The particular mutations are marked as an asterisk within the box representing each particular footprint on the left side of the figure.

Mutations in FPα and FPβ had the most notable effects. Mutation of the ets site in FPα reduced promoter activity in both undifferentiated and differentiated cell to less than 5% of that of the unmutated promoter. The increase in promoter activity with differentiation, however was 11-fold (considerably more than that of the unmutated promoter). Mutation of either the juxtaposed c/ebp or myb sites in FPβ caused a 10-fold reduction in promoter activity in uninduced cells. However the increase of promoter activity of the mutants with differentiation was 8-fold (c/ebp-site mutant) or 11-fold (myb-site mutant) as opposed too only 4-fold for the unmutated promoter. Thus, these mutations of the promoter result in constructs with a similar pattern of an activity as the -105/+15 deletion mutant construct that lacks both the FPβ c/ebp and myb sites.

This fits in with the model proposed above where it is the balance of inhibitors to activators bound to the promoter that is responsible for the overall activity of the construct. These mutants have a low activity due to the loss of activators as opposed to the inclusion of inhibitor sequences which is probably the case with the –180/140 fragment. Mutation of FPγ at either the PU.1-binding or the ETS-binding site had least effect on the *defensin* promoter, both mutations reducing activity to about half in undifferentiated cells and not changing the extent of increase upon differentiation.

With the exception of the FPα ets site mutation, all the other mutant constructs result in a similar promoter activity in differentiated cells. It appears that the loss of one activator set (FPβ- or FPγ-bound) results in a certain reduction in activity as compared to the unmutated promoter (due to a reduction in the activator/inhibitor ratio of one activator). Loss of the FPα Ets-binding site is however more deleterious suggesting that all the other activators can only function in co-operation with the FPα-bound Ets

protein. In undifferentiated cells on the other hand, both the FPα and FPβ-bound factors are essential for good activity such that neither alone can make up for the other. This ties in nicely with the results of the deletion studies where in undifferentiated cells both FPs α and β are required for good promoter activity. It is interesting that none of the FPβ and FPγ site-specific mutant reporters reach the same activity as the -67/+15 deletion mutant in differentiated cells (which is the same as that of the -240/+15 reporter) This suggests that sequences upstream of -67 have a negative effect when unopposed by FPβ or FPγ- binding factors.

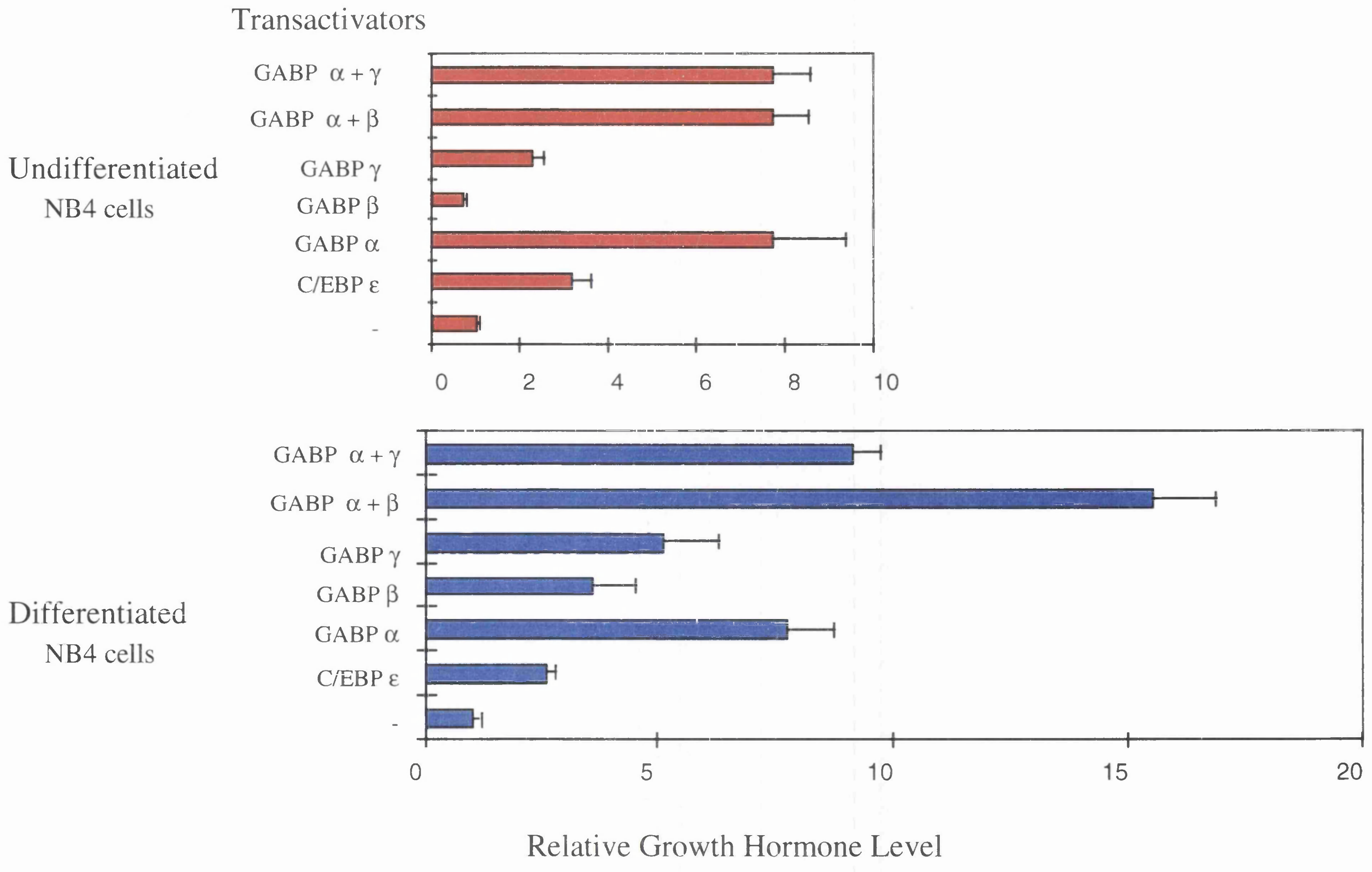
12.3 Co-transfection studies with various activators in NB4 cells

From these previous studies, it was apparent that factors that bind to particular sites within the *defensin* promoter greatly influence its activity. A number of transcription factors were identified by EMSAs to bind the various sites. Constructs expressing these transcription factors were co-transfected together with the -240/+15 *defensin* promoter fragment-reporter gene construct, into NB4 cells to determine the particular effects of each factor in a background milieu ideal for *defensin* expression.

GABPαβ co-expression up-regulates the activity of the -240/+15 *defensin*-reporter construct 8 fold whilst C/EBPε expression produced a 3-fold up regulation in undifferentiated cells (Fig.47 upper panel). Myb, PU.1 and AML-1 were ineffective as transactivators in NB4 cells (not shown). This may be due to their already being present in adequate amounts for maximum *defensin* expression within NB4 cells. Alternatively they may require other co-operative partners which are not present in this cell line at this stage of differentiation in order to transactivate the promoter. C/EBPε and GABPαβ on the other hand appear to be limiting with regards to *defensin* expression. Indeed,

Fig.47 Changes in transactivation and transcription factor synergism with differentiation.

The -240/+15 *defensin* promoter-hGH construct (5μg) was transfected into NB4 cells with pSV2Apap (5μg), constructs expressing C/EBPε (100ng), or GABPs (2μg of each), and pBluescript to a total of 30μg of DNA as described. hGH measurements were normalised to those for alkaline phosphatase, to control for variations in transfection efficiency in undifferentiated cells. Mean growth hormone values from at least four independent transfection experiments are then calculated and expressed relative to the normalised value for the -240/+15 promoter construct in the absence of exogenous factors (taken as 1.0). Growth hormone values for differentiated cells are not being related to those in undifferentiated cells and are therefore also normalised to the alkaline phosphatase internal control. Mean values are calculated from 4 independent transfections, and expressed relative to the value obtained without any co-expressed activators.



Transactivators
Undifferentiated
NB4 cells
GABP α + γ
GABP α + β
GABP γ
GABP β
GABP α
C/EBP ε
-
0
2
4
6
8
10
Differentiated
NB4 cells
GABP α + γ
GABP α + β
GABP γ
GABP β
GABP α
C/EBP ε
-
0
5
10
15
20
Relative Growth Hormone Level

increases and later decreases in C/EBPε expression during granulocytic differentiation of the cell line largely parallel the changes in abundance of *defensin* mRNA (386), Fig. 12. Myb or Ets (GABP or PU.1) co-expression with C/EBPε does not produce co-operative activation , resulting in less promoter activity than the sum of that induced by individual factors (not shown).

12.4 GABP up-regulates the *defensin* promoter diversely, on differentiation

GABP is a bipartite transactivator composed of a DNA-binding but transcriptionally inactive Ets factor (α) and a Notch-related transactivatory factor (β) which is attached to DNA through its interaction with GABPα (314). GABPα expression alone has not been shown so far to transactivate any promoter (278;317); GABPβ co-expression was always required.

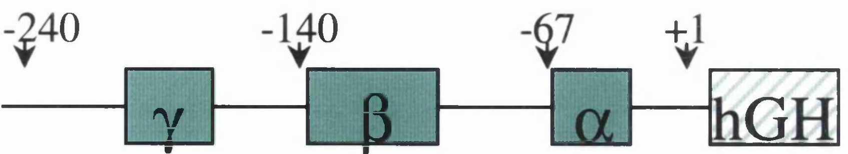
In this study, expression of GABPα alone resulted in similar up-regulation of the *defensin* promoter (8-fold), as did co-expression of both GABPα and β sub-units together, in undifferentiated NB4 cells (Fig. 47 upper panel). Whilst GABPβ alone was completely ineffective in transactivating the *defensin* promoter, GABPγ resulted in a 2 to 3-fold up-regulation of promoter activity. The reason for this difference is unclear. GABPγ, like GABPβ, has a transactivation domain and a domain for heterodimerisation with GABPα. It lacks a homodimerisation domain and is therefore only capable of forming a heterodimer with GABPα but not a heterotetramer (324). Western blot analysis had already shown (Fig. 38) that both GABPα and β are easily detectable in undifferentiated NB4 nuclear extracts, thereby excluding that exogenous expression of GABPα is activating the *defensin* promoter by interacting with endogenous GABPβ which previously lacked a partner.

Unlike the co-transfected GABP sub-units, whose effect on *defensin* promoter activity changed considerably with differentiation, C/EBPε (Fig.47), AML-1 and PU.1 (not shown) expression had a similar effect as in undifferentiated cells. In differentiated NB4 cells, GABPβ (as well as GABPα and GABPγ) co-transfection transactivated the *defensin* promoter. When co-expressed together with GABPα, GABPβ increased *defensin* promoter construct activity in a synergistic manner whilst GABPγ had a less than additive effect when co-expressed with GABPα. Westerns have already shown no great changes in abundance of either GABP sub-unit upon differentiation. EMSA (Fig.25a) suggests binding of GABPβ to FPα in differentiated but not in undifferentiated cells. This suggests a change in the interaction between the two GABP sub-units in differentiated as opposed to undifferentiated cells.

12.5 Effects of site-specific and deletion mutations on transactivation by co-activators

Having identified GABPαβ and C/EBPε as transactivators of the *defensin* promoter in NB4 cells, further studies were performed using different deletion and site-specific mutants in order to identify the important sites through which these factors are acting. The results of these transfections into differentiated NB4 cells are presented in Fig.48. The activity induced by C/EBPε co-transfection with the -67/+15 promoter construct was half that seen with the -240/+15 construct. C/EBPε co-transfection with the FPβ c/ebp site promoter mutant similarly resulted in half the activity of the unmutated -240/+15 promoter construct. This is consistent with the evidence from recent studies on the rat *defensin* gene promoter (as well as my own observations) that

Fig. 48 Transactivation of mutated *defensin* gene promoters in uninduced NB4 cells by GABPαβ and C/EBPε. The cells were transfected with 5μg of the promoter reporter construct, expression constructs (100ng of C/EBPε or 2μg each of GABPα and GABPβ), 5μg of pSV2Apap, and pBluescript to 30μg. hGH measurements were normalised to those for alkaline phosphatase to control for variations in transfection efficiency. The means of four independent transfection experiments relative to that for the -240/+15 promoter construct transactivated by C/EBPε (taken as 1.0) are shown.


-240
-140
-67
+1
γ
β
α
hGH

Transfections
-240 γ-mut-ets + GABPαβ
-240 α-mut-ets + GABPαβ
-67 + GABPαβ
-240 + GABPαβ
-240 α-mut-ets + C/EBPε
-240 β-mut-myb + C/EBPε
-240 β-mut-c/ebp + C/EBPε
-67 + C/EBPε
-240 + C/EBPε
0
0.5
1
1.5
2
2.5
3
Relative Growth Hormone Level

C/EBPs also bind within the gene's -67/+15 sequence (53). Therefore, only half the functional c/ebp sites would be lost by mutation or deletion of FPβ. The -240/+15 construct lacking the FPβ myb site, however, showed 4-fold lesser C/EBPε-induced activity compared to the unmutated construct. This is considerably less than the activity seen with the FPβ c/ebp site mutated construct suggesting that binding of c-Myb is very important for *defensin* promoter activity.

Interestingly, C/EBPε-induced activity was completely lost with mutation of the GGAA core of the ets site in FPα (Fig.48), suggesting that binding of an Ets factor (probably GABP) is essential for C/EBPε-dependent activation of the promoter. As indicated from previously presented transfected studies with mutant reporter genes, the FPα-bound factors appear essential for any *trans*-activation of the *defensin* promoter. Co-expression of GABPα and β transactivated the minimal -67/+15 promoter construct and the longer -240/+15 construct to a similar extent, suggesting that the majority of GABP-induced activity was dependent on the FPα ets site. Mutation of the core GGAA in FPα resulted in a 5-fold reduction in GABPαβ-induced activation, confirming the importance of this binding site. Mutation of the core ets site in FPγ (Fig.48) reduced GABPαβ-induced activation only minimally suggesting that any GABP binding we may have noted at this site is either an artefact of the EMSA or is functionally irrelevant *in vivo*.

Chapter 13 : Promoter studies in non-myeloid HeLa cells

Many transcription factors present in NB4 cells may play a role in *defensin* promoter transactivation. Therefore, in order to observe any co-operative transactivation effects in a cell line with low background levels of many of the factors involved, functional studies were also performed in non-myeloid HeLa cells which do not express *defensin*. The -240/+15 *defensin* promoter construct had negligible activity in HeLa cells (Fig.49). The -140/+15 construct had a similarly negligible activity though that of the -67/+15 construct was marginally increased in relation to the longer constructs. This is possibly due to interaction of different endogenous factors (such as GABPα and C/EBPβ) on this short sequence resulting in some synergism (evidence for such an interaction is shown later). On longer constructs, these factors would most likely have been bound to separate sites and would be less likely to interact.

13.1 Myb and C/EBPε synergistically transactivate the *defensin* promoter

Co-transfection of a Myb-expressing construct did not increase the basal promoter activity of the –240/+15 reporter construct in HeLa cells (Fig.49). C/EBPε co- expression produced only a three-fold up-regulation whilst co-transfection of C/EBPε together with Myb produced a clearly synergistic 26-fold activation. Similar co-operative transactivation was apparent with the -140/+15 promoter construct but there was no such co-operative transactivation of the -67/+15 promoter. This correlates

Fig. 49 Transactivation of *defensin* gene promoters in HeLa cells by MYB and C/EBPε.

The cells were transfected with promoter construct (5μg in each case), expression constructs (100ng of C/EBPε and/or 2μg of Myb as indicated), and pBluescript to 10μg. Since no internal control plasmid is being used (due to the fact that it was markedly effected by some co-expressed transactivators), it is essential that multiple repeats of each experiment be performed. For this reason, for each experiment the means of four independent transfections are calculated and expressed relative to that for the -240/+15 promoter construct in the absence of exogenous factors (taken as 1.0).

-240
-140
-67
+1
γ
β
α
hGH
Transfection
-240 γ-mut-ets + MYB + C/EBPε
-240 α-mut-ets + MYB + C/EBPε
-240 β-mut-myb + MYB + C/EBPε
-240 β-mut-c/ebp + MYB + C/EBPε
-67 + MYB + C/EBPε
-140 + MYB + C/EBPε
-240 + MYB + C/EBPε
-240 + C/EBPε
-240 + MYB
-240 β-mut-myb
-140
-67
-240
0
0 5 10 15 20 25 30 35 40
Relative Growth Hormone Level

with the absence of the FPβ myb site in the latter. Using site-specific mutant promoter constructs we analysed the binding site requirements for this co-operative transactivation. The FPβ c/ebp-site mutant was transactivated just as strongly by C/EBPε and Myb as the unmutated construct whilst the FPβ myb-site mutant showed markedly reduced transactivation, indicating an absolute requirement for a Myb-binding site. Strong transactivation of the FPβ c/ebp-site mutant promoter construct suggests that Myb is capable of co-operating with C/EBP bound elsewhere on the promoter (possibly at FPα), and indicates that myb and c/ebp binding sites need not necessarily be adjacent for co-operative transactivation of the promoter.

The -240/+15 promoter construct mutated at the FPα ets site was not transactivated by co-expression of C/EBPε and Myb, indicating that this site is essential for any transactivation by C/EBP just as was seen with transfections into NB4 cells. On the other hand, the promoter construct bearing an ets site mutation in FPγ, was co-operatively transactivated by C/EBPε and Myb to a similar extent as the unmutated construct. The importance of the FPα Ets-binding site is emphasised even in this cell line indicating that the factor bound here is most probably not a myeloid-specific factor. Alternatively, the function of whatever binds here in myeloid cells can be effectively substituted in non-myeloid ones.

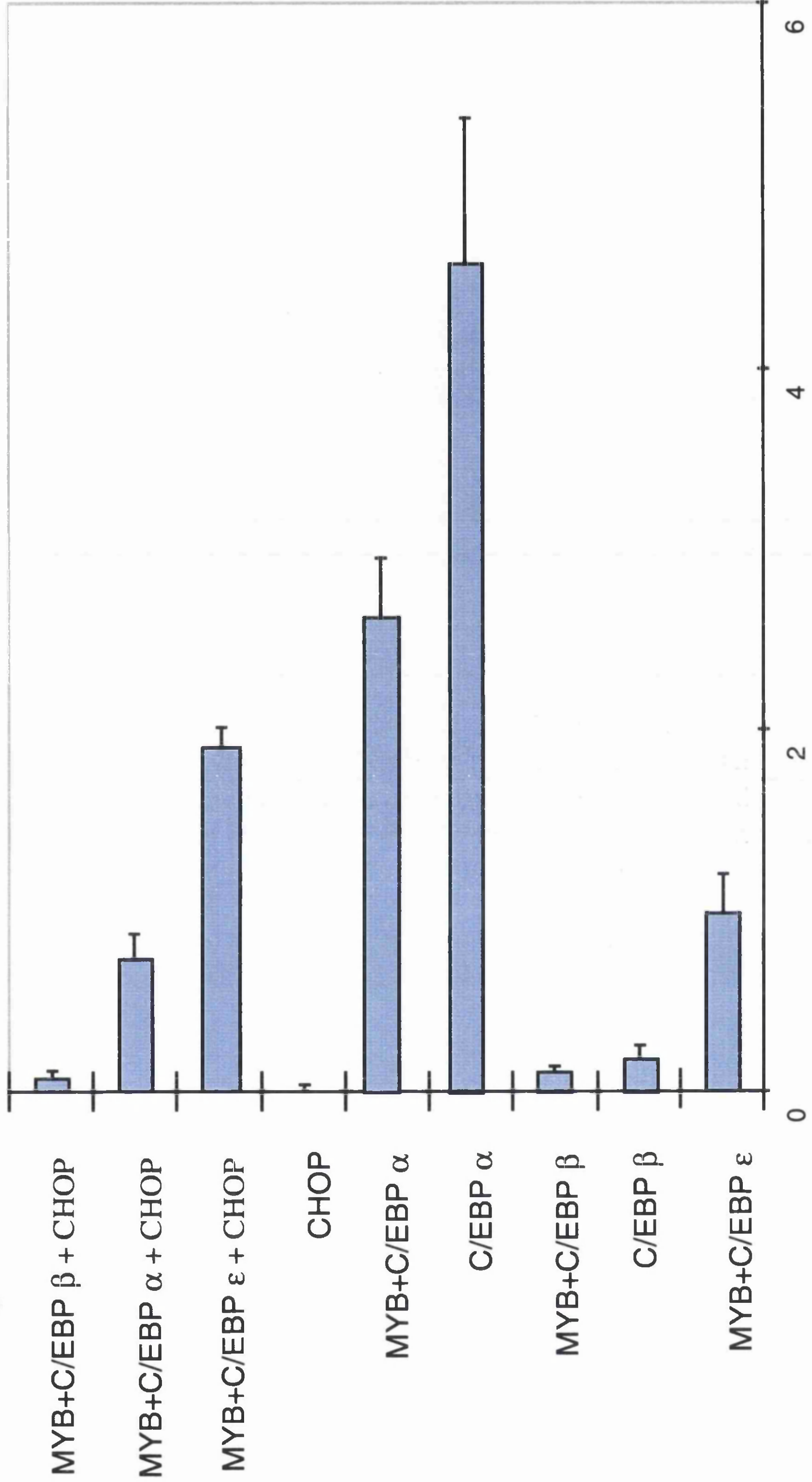
13.2 C/EBP α and β do not co-operate with c-Myb and can be inhibited by CHOP-10, unlike CEBPε

The main transactivating C/EBP family member found in HeLa cells is C/EBPβ (145). As expected from the low background activity of the *defensin* promoter in HeLa

cells, overexpression of C/EBPβ did not produce any increase in reporter activity, nor was there any significant co-operative transactivation with Myb (Fig.50). Co-expression of C/EBPα with Myb resulted in transactivation three-fold greater than that observed with C/EBPε and Myb. C/EBPα alone was, however, an even more potent transactivator of the *defensin* promoter so that Myb actually reduced C/EBPα-induced transactivation. When CHOP-10 (154), a dominant negative form of C/EBP, was co-transfected together with the various C/EBP family members and Myb, it inhibited C/EBPα or β-induced transactivation but enhanced transactivation by C/EBPε and Myb. These differences probably have to do with the different structures of the different C/EBP family members particularly in the leucine zipper domain. C/EBPε is poor at dimerisation through its leucine zipper and unlike the others may bind DNA predominantly as a monomer (153;370). These differences probably explain why C/EBPε is protected from inhibition (due to CHOP-10 induced disruption of DNA binding). C/EBPε's poor heterodimerisation may also be responsible for its better co-operation with c-Myb since Myb is known to interact with C/EBP family members through their basic zipper domains (235). This interaction is essential for functional co-operation, so not having to compete with other C/EBP dimerisation partners is possibly beneficial to interaction of Myb with C/EBPε. The increased transactivation caused by the co-transfection of CHOP-10 with C/EBPε and Myb is probably due to disruption of endogenous C/EBPβ binding to DNA thereby allowing enhanced C/EBPε binding, to sites previously occupied by C/EBPβ.

Myb has however been shown to co-operate with C/EBPα and β in other studies (218;235), suggesting that the conditions of each particular experiment (including the cell background and possibly the expression vectors used) may play a role in such co-operativity or the lack of it.

Fig50 Effects of CHOP on Myb-C/EBP-induced transactivation of the *defensin* promoter. The defensin –240/+15 promoter-hGH construct (5μg) was transfected into HeLa cells with constructs expressing transcription factors as indicated: 100ng of C/EBPα, β or ε, 100ng of CHOP-10, 2μg of c-Myb; and pBluescript to 10μg. The mean growth hormone level from four independent transfection experiments is calculated in each case and expressed relative to that calculated for co-expression of C/EBPε + MYB with the reporter (taken as 1.0).



Transfected Transcription Factors
MYB+C/EBP β + CHOP
MYB+C/EBP α + CHOP
MYB+C/EBP ε + CHOP
CHOP
MYB+C/EBP α
C/EBP α
MYB+C/EBP β
C/EBP β
MYB+C/EBP ε
0
2
4
6
Relative growth hormone level

13.3 GABPα strongly activates the *defensin* promoter without GABPβ

GABPβ and γ both produced a 10 to 20-fold transactivation of the -240/+15 *defensin* reporter construct in HeLa cells, but GABPα alone, transactivated the promoter 100-fold. The empty GABP expression vector pCAGGS had no effect on *defensin* promoter activity (Fig.51). Transactivation of this promoter by GABPα alone was similarly seen in NB4 cells, though not to such a great extent. In HeLa cells, however, unlike in NB4 cells, co-transfection of GABPβ together with GABPα resulted in an 80% reduction in reporter activity compared to that with GABPα alone. GABPβ produced a transactivation similar to that of GABPαβ, possibly by enhancing translocation of endogenous GABPα into the nucleus. It has been shown that GABPα is not strongly localised in the nucleus in the absence of a dimerisation partner (317). It is noteworthy that GABPγ is considerably less inhibitory than GABPβ when co-expressed in HeLa cells with GABPα, suggesting that protein domains which differ between GABPβ and γ are partially responsible for this inhibitory effect. GABPα induced the activities of the -140/+15 and the -240/+15 constructs to about the same extent, but induced that of the -67/+15 deletion mutant to a lesser extent (Fig.51). This indicates that sequences upstream of the FPα ets site are required for optimum GABPα-induced activity unilke in NB4 cells. FPβ, that was shown to bind GABPα may be such a sequence (see Fig.28d).

Transfections of GABPα together with site-specific mutant promoter constructs were performed to identify the sequences involved in GABPα-induced transactivation. This was completely lost in the case of the FPα core GGAA ets site mutant, as was

Fig.51 Transactivation of *defensin* mutant promoter constructs in HeLa cells by GABPs.

Cells were transfected with 5μg of promoter construct, GABP-expression constructs or empty expression vector, pCAGGS (2μg of each vector transfected), and pBluescript to 10μg. The mean growth hormone level of four independent transfection experiments was calculated and expressed relative to that measured for the -240/+15 promoter construct transactivated by GABPα and β (taken as 1.0).

Transfections

-240 + p CAGGS
-240 γ-mut-pu.1+ GABPα
-240 γ-mut-ets + GABPα
-240 β-mut-c/ebp + GABPα
-240 α-mut-ets + GABPα
-67 + GABPα
-140 + GABPα
-240 + GABPαγ
-240 + GABPαβ
-240 + GABPα
-240 + GABPγ
-240 + GABPβ

0 2 4 6 8 10 12

Relative Growth Hormone Level

expected due to this site being essential (as previoulsy shown) for any promoter activity. It has already been shown to bind GABP (Fig. 25a). Mutation of the c/ebp site in FPβ and of the core ets site in FPγ also reduced GABPα-induced transactivation about 4-fold (Fig.51). A mutant construct where the FPγ core GGAA was unmutated but the adjacent nucleotides had been modified to inhibit PU.1 binding, actually resulted in even greater transactivation by GABPα. This is probably because an additional Ets-binding site similar to the consensus GABPα binding site had been created by such modifications.

It is particularly interesting in these mutagenesis studies that the mutation of specific sites within footprints was often more deleterious to GABPα-induced activation than deletion of the entire footprint (eg. 240/+15 γ-mut-ets as opposed to –140/+15). This reduced transactivation may be due to disruption of paired sites where GABP and other co-operative factors (possibly C/EBP) bind adjacently. This may result in the transcription factors binding to preferred sites separated along the length of the promoter rather than adjacently, thereby reducing functional co-operation.

GABPα is an Ets factor and is rather similar in structure to various other members of the Ets family (including Ets-1, Ets-2 and TEL). Ets-2 - another Ets factor known to be expressed in haematopoietic cells; and quite ubiquitously (389) was co-transfected with the *defensin* reporter genes. This resulted in only a 4-fold activation of the promoter, similar to the transactivation seen by PU.1, indicating that massive transactivation by GABPα is not a non-specific Ets-induced effect (See Fig.53)

In order to try and understand why GABPα was seen to have this effect in our system, an experiment was performed using a previously studied promoter which GABPα did not up-regulate when transfected into HeLa cells. To identify any influences due to the novel reporter system (most transfection studies with GABP have

used a luciferase-based reporter vector of some sort), I cloned the *cd18* minimal promoter sequence into my reporter vector and transfected this construct into HeLa cells together with the different GABP expression constructs. The results I obtained (Fig. 52) were similar in pattern if not in degree to those seen with the *defensin* promoter. GABPα co-transfection resulted in transactivation, unlike the combination of GABPαβ which was inactive and where GABPβ and GABPγ actually repressed basal promoter activity. This suggests that our reporter system is influencing the results obtained. A possible explanation for these differences in the reporting systems and the significance/not of our results is proposed later (see section 15.4).

13.4 PU.1 co-operates with GABPαβ but not with GABPα in transactivating the *defensin* promoter

It has been shown that GABPαβ and PU.1 co-operate in transactivating the *cd18* promoter. Both these factors can bind to the *defensin* promoter (Figs.25a, 31a), so HeLa cells were co-transfected with GABPαβ and PU.1 expressing vectors. The combined Ets factors transactivated the -240/+15 reporter construct more than the sum of each individual factor (Fig. 53). The -140/+15 construct, though showing reduced PU.1- and GABPαβ-induced activation, was strongly synergistically transactivated by the two factors together. This suggests that the FPγ ets site may not be essential for this co-operativity. Transactivation of the –67/+15 promoter construct by PU.1 and GABPαβ was less than additive, possibly due to a degree of competition for the same FPα ets

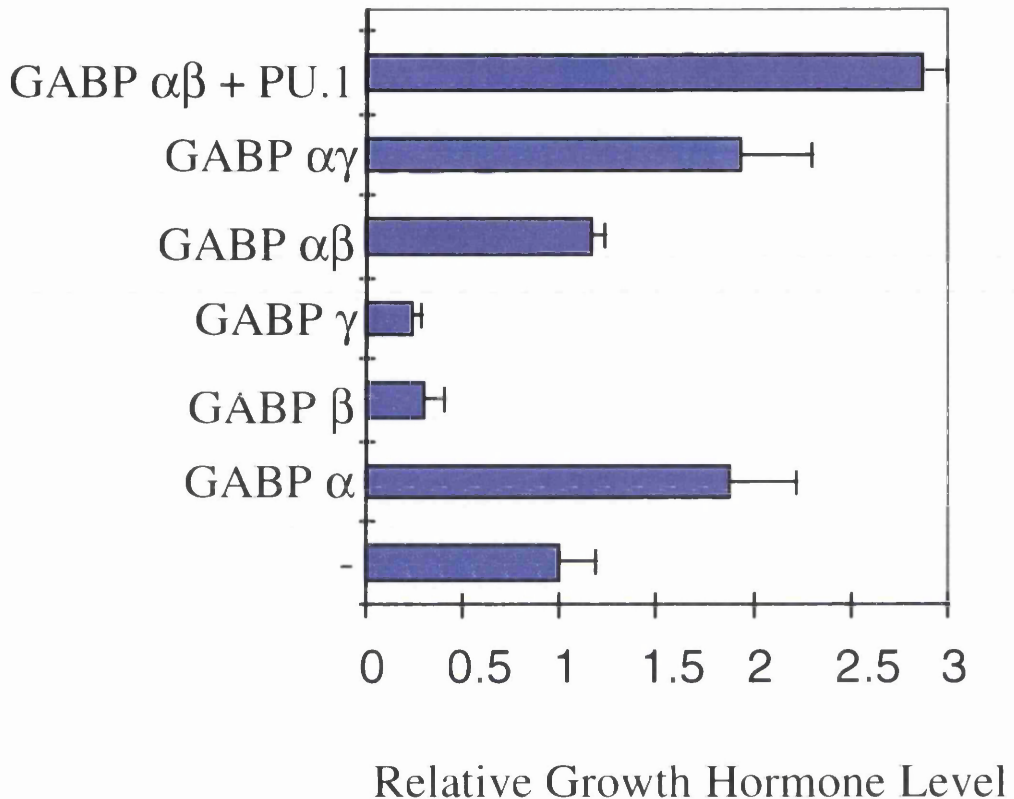
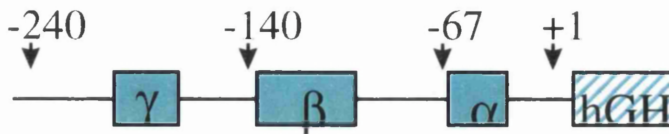


Fig.52 Transactivation of the *cd18* minimal promoter (cloned into the final growth hormone reporter vector) by GABP factors.

HeLa cells are transfected with the minimal *cd18* promoter-hGH construct (5μg) together with GABP expression vectors (2μg of each) and carrier plasmid up to 10μg of total DNA per transfection. PU.1-expression vector (1μg) is co-transfected in one case. Growth hormone levels are measured, means of three independent experiments are calculated and values expressed relative to that measured after transfection of the minimal promoter without co-expressed activators.

Fig.53 Co-operative transactivation of *defensin* mutant promoter constructs in HeLa cells by PU.1 and GABP$\alpha\beta$.

The cells were transfected as indicated to the left of the bars, with 5µg of each promoter construct, expression constructs (1µg of PU.1 or PU.1-N.133; 2µg of each GABP; 500ng of Ets-2) or empty expression vector, pcDNA (1µg), and pBluescript to 10µg. The mean growth hormone levels of four independent transfections was calculated and expressed relative to that for the -240/+15 promoter construct transactivated by GABPα and β(taken as 1.0).



Transfections

-240 + ETS-2
-240 + GABPα + pcDNA
-240 + PU.1(N133) + GABPα
-240 + PU.1 + GABPα
-240 + GABPα
-240 γ-mut-ets + GABPαβ + PU.1
-240 γ-mut-ets + PU.1
-240 γ-mut-ets + GABPαβ
-140 + PU.1 + GABPαβ
-140 + PU.1
-140 + GABPαβ
-67 + PU.1 + GABPαβ
-67 + PU.1
-67 + GABPαβ
-240 + PU.1(N133) + GABPαβ
-240 + PU.1 + GABPαβ
-240 + PU.1
-240 + GABPαβ

0 1 2 3 4 5 6 7

Relative Growth Hormone Level

site. Therefore whilst FPγ is not important for co-operativity, sites upstream of –67 (possibly FPβ which can bind GABPα) are required.

These experiments suggest that possibly GABP$\alpha\beta$ and PU.1 co-operativity is not solely dependent on direct PU.1 binding to DNA. To study this, a deletion mutant of PU.1 (N-133), which binds DNA at least as strongly as PU.1 (See Fig. 31b) but which lacked PU.1 transactivation domains, was used. When transfected together with GABP$\alpha\beta$ into HeLa cells, PU.1 mutant (N-133) markedly inhibited GABP$\alpha\beta$-induced transactivation. This confirms that PU.1 sequences outside the DNA-binding domain are essential for the co-operative promoter transactivation together with GABP$\alpha\beta$.

Unlike the co-operative effect between GABP$\alpha\beta$ and PU.1, transactivation by GABPα alone was markedly inhibited by PU.1 (Fig. 53). This inhibition is possibly due to binding-site competition between the two Ets factors for the same site, as has been shown to occur on other promoters (322). The PU.1 (N133) mutant produced a similar inhibitory effect, confirming that this is likely to be due to DNA binding-site competition (Fig.53).

Though PU.1 and GABP$\alpha\beta$ co-operate in transactivating *defensin* promoter constructs, further increases in the amount of PU.1, once again reduced the level of transactivation (Fig. 54). This inhibition is not produced by increasing the amount of co-transfected pcDNA empty expression vector and is therefore not due to squelching by the pcDNA vector promoter. Excess PU.1 may inhibit GABP$\alpha\beta$ binding to DNA due to a competitive effect as seen with GABPα.

A possible explanation as to the cause of the co-operativity between GABP and PU.1 can be derived from the EMSAs showing competitive binding on FPα in NB4 cells (see Fig.37). In these experiments, removal of PU.1 binding by a competitive oligonucleotide resulted in enhanced C/EBPε binding whilst competing away any

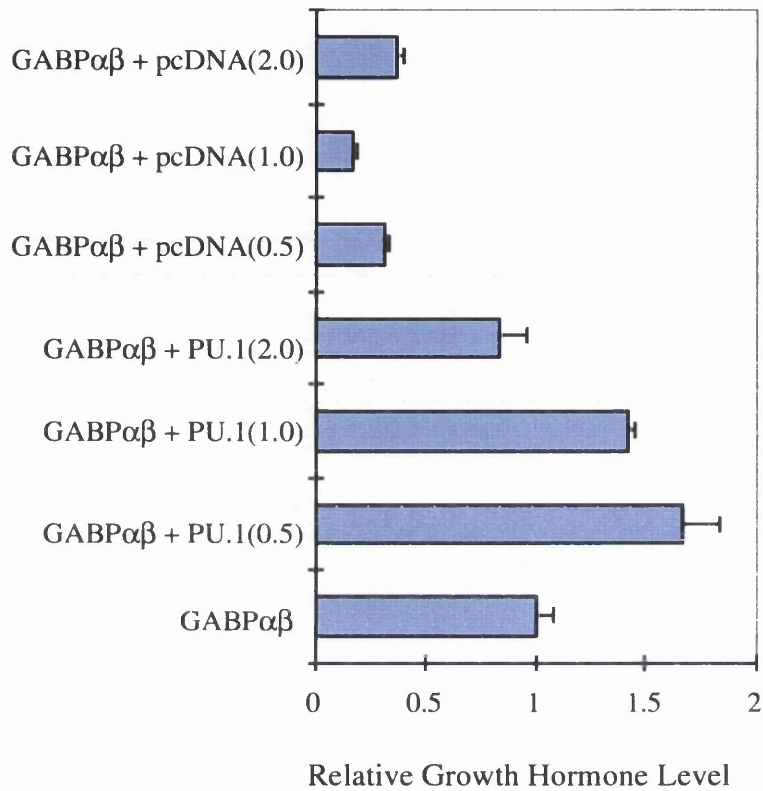


Fig.54 Increased amounts of PU.1-expressing vector repress GABPαβ-induced transactivation.
HeLa cells are transfected with –240/+15 reporter construct, GABP α and β expression vectors (2μg of each) and increasing amounts of PU.1-expression vector or empty expression vector pcDNA (amount in μg, indicated to the left of the bar graph). Mean growth hormone values from 4 separate experiments are calculated and expressed relative to that for GABPαβ expression alone with the reporter (taken as 1.0).

C/EBP binding to FPα, resulted in a number of clear bands indicating complexes bound to FPα, one of which seemed to be include GABPβ. Since removal of PU.1 enhances C/EBP binding, addition of extra PU.1 will probably further reduce C/EBP binding to FPα. C/EBPβ might inhibit GABPαβ transactivation either by competing with the GABP heteromer for FPα binding, or by interacting with the GABP sub-units thereby inhibiting their interaction and co-operative DNA binding. Data presented further on supports this second hypothesis. PU.1, by interacting with C/EBPβ, as has been recently shown (239), may disrupt interaction of this protein with the GABP sub-units, thereby allowing the latter to co-operatively interact.

The requirement for promoter sequences upstream of FPα for PU-1-GABP co-operation to occur suggests that the PU1-C/EBPβ complex can compete for the same FPα site. This might prevent GABPαβ binding and transactivation unless there are alternative binding sites (such as possibly FPβ). Addition of more PU.1 will compete with the GABPαβ dimer for FPα binding therefore reversing the co-operative effect of a smaller dose of PU.1.

13.5 Additive and co-operative transactivation of the *defensin* promoter by different transcription factors

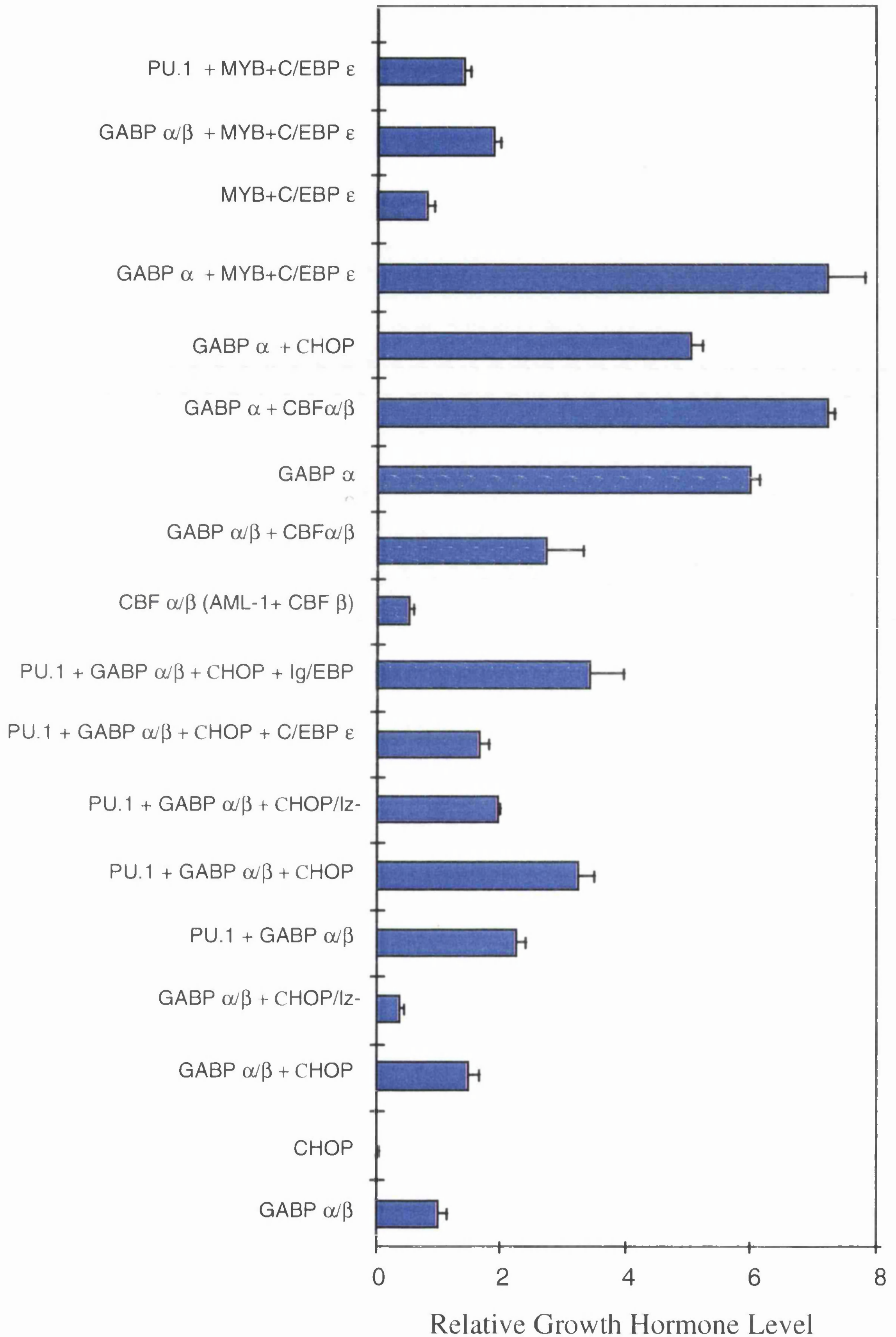
CHOP-10 heterodimerises with many of the C/EBP family proteins and disrupts their binding to DNA thereby inhibiting C/EBP-dependant transactivation (154). CHOP-10, enhances GABPαβ-induced activation whilst co-transfection of exactly the same vector with a leucine zipper mutation in CHOP-10 (making it incapable of dimerisation) does not. It actually reduces GABPαβ transactivation somewhat, possibly

by promoter competition as a result of the expression vector promoters squelching essential basal transcription factors (Fig. 55). CHOP had no such co-operative effect when co-transfected with GABPα alone, suggesting that, as in the case of PU.1-dependant co-operativity, only GABPαβ heteromer-dependant transactivation is enhanced. CHOP-dependant enhancement of GABPαβ promoter transactivation is also seen in the presence of PU.1. This co-operativity (CHOP-PU.1-GABPαβ) was inhibited by the addition of C/EBPε but not by the inhibitory C/EBP factor Ig/EBP (C/EBPγ).

CHOP is an inhibitory factor which heterodimerises with C/EBP factors through their leucine zipper domain and inhibits their binding to DNA due to its own basic domain being defective in DNA-binding (154). Co-expression of this inhibitory factor causes an increase in GABPαβ-dependant promoter activation, just as it was seen to increase the activation of the promoter by C/EBPε and MYB. This suggests that CHOP is allowing GABPαβ to bind to DNA sites uninhibited by some C/EBP factor (most probably C/EBPβ), and to transactivate the promoter. C/EBPε (which heterodimerises poorly with many C/EBPs (153)) might therefore not interact with CHOP-10 and can inhibit GABPαβ in C/EBPβ's place. The ability of C/EBPε to block the CHOP-GABPαβ synergism supports this hypothesis. Ig/EBP, though normally inhibitory, dimerises well and should therefore also interact with CHOP, which would explain its inability to block the synergism.

One other factor known to be involved in the expression of many early myeloid genes (185), is AML-1B (CBF-α). There is also evidence of this factor being capable of *defensin* transactivation (53). Co-transfected into HeLa cells with its partner CBFβ, it can activate the *defensin* promoter and is clearly co-operative with GABPαβ (Fig. 55). It also enhances GABPα-dependent transactivation though in an additive, not synergistic, fashion. AML is also known to interact with C/EBPα, bind

Fig.55 Co-operative transactivation of the defensin promoter in HeLa cells by different sets of transcription factors. The d*efensin* –240/+15 promoter-hGH construct (5μg) was transfected into HeLa cells with constructs expressing transcription factors as indicated: 100ng of each C/EBP including CHOP, the leucine zipper mutant of the latter factor CHOP-lz and the inhibitory C/EBP family member Ig/EBP, 1μg of PU.1, 2μg of MYB, 2μg of each GABP, 100ng of CBFα, 100ng of CBFβ, and pBluescript to 10μg.The mean expressed growth hormone levels for each experiment are calculated from four independent transfections and are presented relative to that obtained with co-expression of GABPαβ (taken as 1.0).



Transfected transcription factors
PU.1 + MYB+C/EBP ε
GABP α/β + MYB+C/EBP ε
MYB+C/EBP ε
GABP α + MYB+C/EBP ε
GABP α + CHOP
GABP α + CBFα/β
GABP α
GABP α/β + CBFα/β
CBF α/β (AML-1+ CBF β)
PU.1 + GABP α/β + CHOP + Ig/EBP
PU.1 + GABP α/β + CHOP + C/EBP ε
PU.1 + GABP α/β + CHOP/lz-
PU.1 + GABP α/β + CHOP
PU.1 + GABP α/β
GABP α/β + CHOP/lz-
GABP α/β + CHOP
CHOP
GABP α/β
0
2
4
6
8
Relative Growth Hormone Level

co-operatively with it, to the DNA(238). It may therefore enhance GABPαβ-dependent transactivation by a different mechanism than that hypothesised for CHOP-10. AML-1 by co-operatively binding to the DNA with C/EBP at tandem aml-c/ebp sites elsewhere along the promoter would allow GABPαβ to bind to FPα and transactivate the promoter. This mechanism may be somewhat similar to that suggested for PU.1.

C/EBPε and Myb, when co-transfected together with GABPα or with GABPαβ, increase *defensin* promoter activity additively (Fig.55). Myb and C/EBPε also enhance reporter activity in an additive fashion when co-transfected with PU.1. This suggests that C/EBPε binds independently and to separate sites from the Ets factors when in the presence of Myb without influencing their ability to transactivate the promoter.

Chapter 14 : *In vitro* binding studies with HeLa nuclear proteins

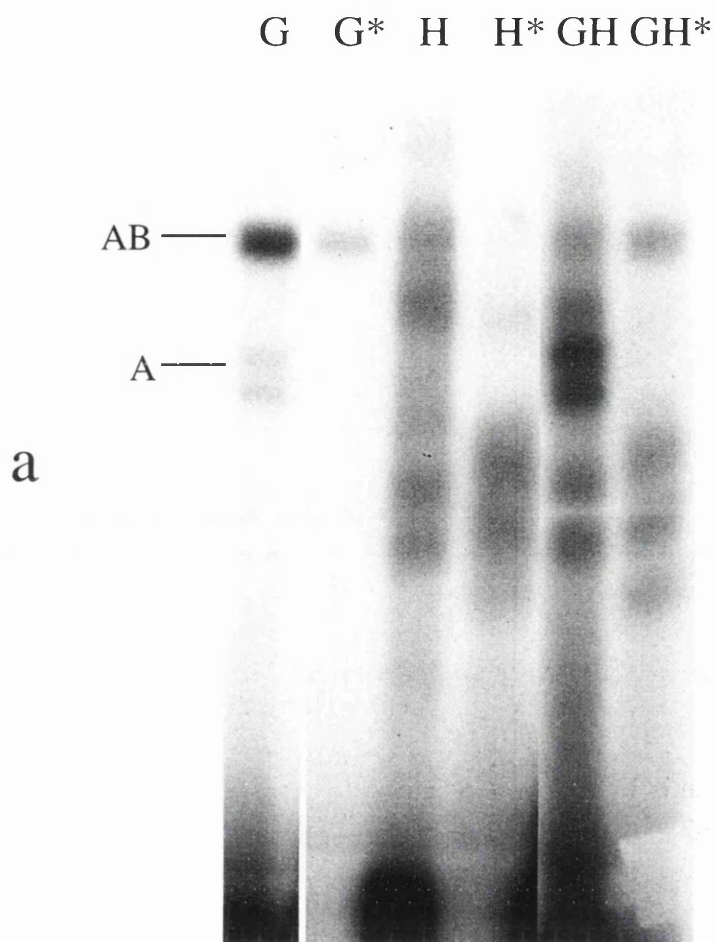
In order to try and understand the mechanisms involved in *defensin* promoter transactivation in HeLa cells, some EMSAs were performed.

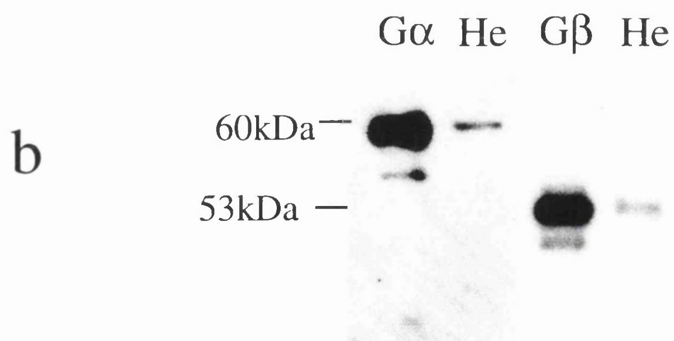
14.1 GABP proteins in HeLa cells -Westerns and EMSAs

As in myeloid NB4 cells, western blotting of HeLa nuclear proteins was performed primarily to exclude that transfected GABPα was co-operating with endogenous GABPβ which lacked a dimerisation partner (endogenous GABPα). This was clearly however not the case (Fig. 56b).

Using *defensin* FPα and the Ets-binding site from the *neutrophil elastase* promoter as labelled probes, HeLa extracts were incubated with or without purified GABP proteins. As can be seen in Fig. 56a, purified GABP proteins bind more strongly both as GABPα monomers and as a heteromer to the FPα site. GABP binds to the *neutrophil elastase* promoter ets site only as an αβ dimer. HeLa nuclear extract binds to both probes but a band of similar mobility as the GABPαβ dimer is visible only with the FPα probe. On addition of purified GABPα and β proteins to the nuclear extract, an important difference emerges. Whilst the αβ heteromer is still the primary form bound to the NE probe (if anything it is more strongly bound in the presence of extract), GABP binds mostly in bands indicative of GABPα alone to the FPα probe whilst the strong GABPαβ heteromer binding is absent. This therefore supports the hypothesis that in

Fig. 56 GABP proteins in HeLa cells and their binding to the defensin promoter.
(a) 25ng of purified proteins GABPα and GABPβ (G) 10μg of HeLa nuclear extract (H), or HeLa extract with GABP proteins (GH) are incubated with labeled FPα probe in the presence of buffer 3. These sets of proteins are similarly incubated in other reactions also in buffer 3 with the radiolabeled ets site from the *neutrophil elastase* promoter as probe (G*, H* and GH*). Complexes formed are separated by electrophoresis through a 4.5% polyacrylamide gel with a 0.25xTBE buffer to allow good GABP binding. Bands A and AB identify the complexes made by GABPα alone and the GABPαβ dimer respectively with FPα.
(b) Western blotting analysis of 10μg of HeLa nuclear extract (He) and 25ng of purified GABPα (Gα) and GABPβ(Gβ). Following incubation with anti-GABP primary antibodies, protein is detected by chemi-luminescence using an-HRP-linked secondary antibody. Both GABP proteins are clearly well expressed in HeLa cells.


G G* H H* GH GH*
AB
A
a


Gα He Gβ He
60kDa
53kDa
b

the presence of HeLa nuclear factors, the GABPαβ heteromer does not bind strongly to FPα apparently because some factor within the HeLa nuclear extract binds to GABPβ preventing it from interacting with GABPα. The same HeLa extract, however, does not disrupt the GABPα-GABPβ interaction and binding when added to the *neutrophil elastase* (NE) Ets-binding site. One possible explanation for this difference is that strong GABPα binding to the DNA is required for this other factor to interact with GABPβ in GABPα's place.

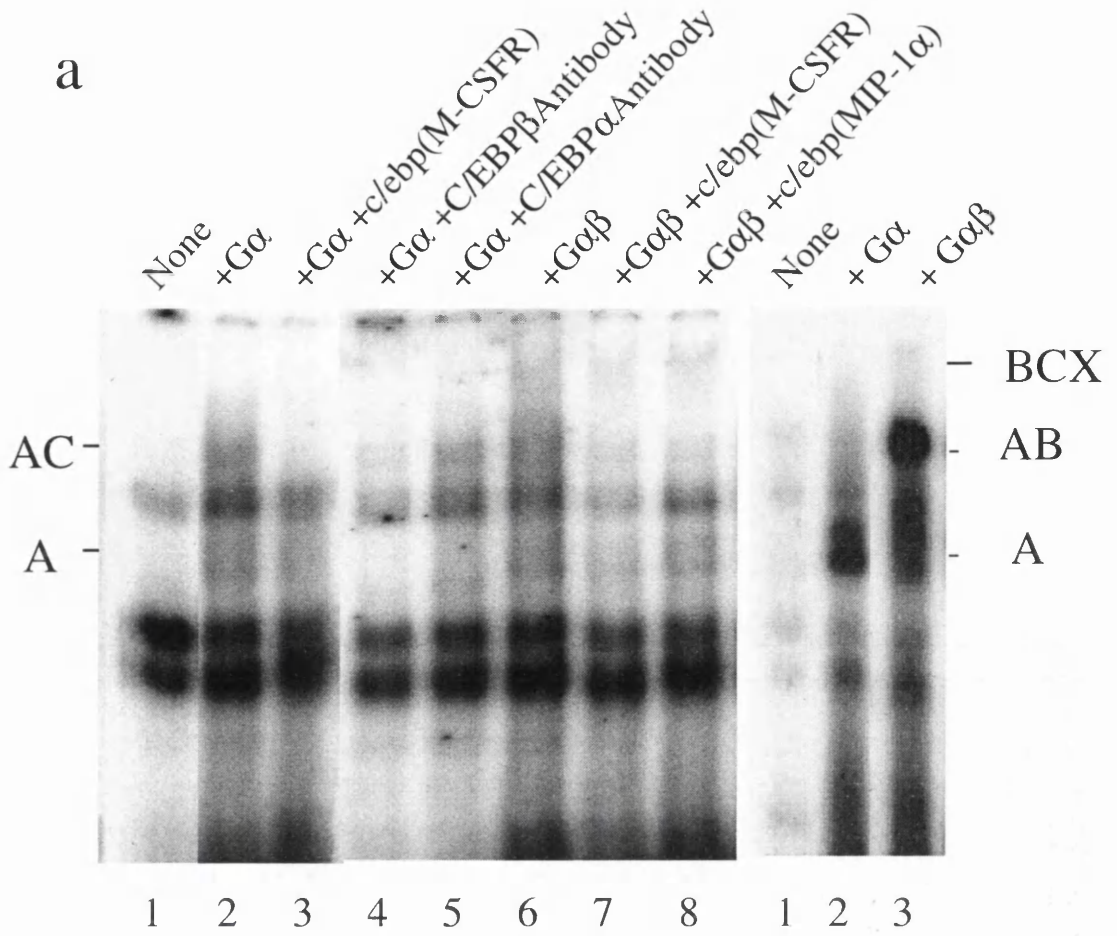
14.2 GABPα forms a complex other than GABPαβ with HeLa extract

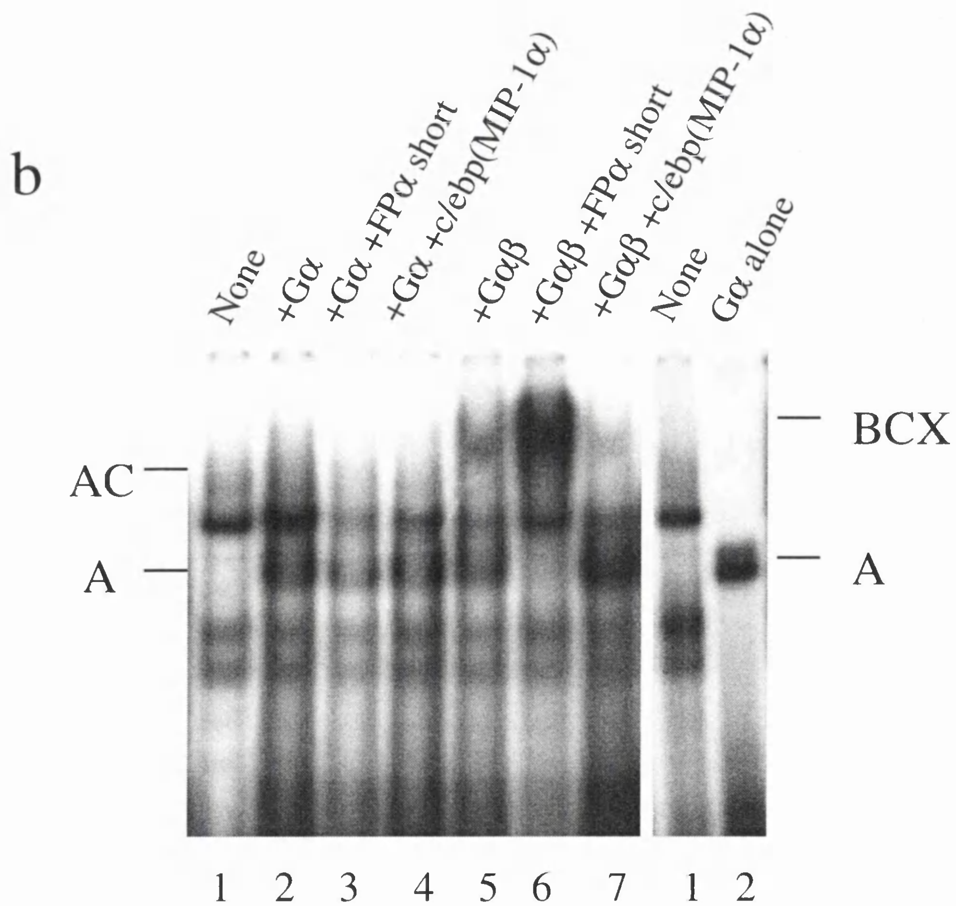
EMSAs where purified GABPα (25ng-0.75μl) was added to HeLa nuclear protein extract (5μg 2μl) and FPα probe (Fig. 57a) showed the formation of at least 2 new complexes marked as band A (GABPα alone-see Fig.57b-right panel) and band AC (GABPα with another factor/s). As can be seen, the AC band can be competed away specifically by a C/EBP binding site oligonucleotide from the *m-csf Receptor* promoter. Anti-C/EBPβ antibody, but not anti-C/EBPα antibody also reduces binding of this complex. GABPα binding (band A) is also inhibited by both antibodies (possibly a non-specific effect). Addition of GABPβ (25ng-0.75μl) together with GABPα results in the formation of a very low mobility complex marked as BCX. The intensity of this band as well as that of the AC band was mildly reduced by two different C/EBP-binding oligonucleotides from different promoters. One of these oligonucleotides also reduced band A intensity.

Fig.57 Formation of complexes other than GABPαβ between GABP sub-units and HeLa nuclear proteins.

(a) 5μg of HeLa nuclear protein extract are incubated together with labeled FPα probe and purified GABPα (Gα) and/or GABPβ (Gβ) proteins (25ng), various oligonucleotide competitors and antisera (as indicated) in buffer 1. Complexes formed by these incubations are separated by electrophoresis through a 6% polyacrylamide gel with a 0.5xTBE buffer. The panel on the right on the other hand represents 5μg of HeLa nuclear extract incubated with 25ng of purified GABP proteins (as indicated) in buffer 3 and separated by electrophoresis through a 6% polyacrylamide gel with a 0.25xTBE buffer to enhance GABP binding. It is interesting to note that unlike Fig 56 where there was a greater HeLa extract: GABP ratio, here, GABPαβ binds the DNA as a dimer despite the presence of HeLa proteins. Complexes A and AB, as in figure 56 indicate the likely position of the GABPα-DNA and GABPαβ-DNA complexes. Complex AC is a complex apparently consisting of GABPα and C/EBPβ, whilst band BCX identifies a complex containing GABPβ and C/EBPβ and probably some other factors.

(b) 5μg of HeLa nuclear protein extract are incubated together with labeled FPα probe and purified GABP proteins (25ng), various oligonucleotide competitors and antisera (as indicated) in buffer 1. Bands A, AC and BCX represent the same factor complexes described in Fig 57(a).


a
None
+Gα
+Gα +c/ebp(M-CSFR)
+Gα +C/EBPβAntibody
+Gα +C/EBPαAntibody
+Gαβ
+Gαβ +c/ebp(M-CSFR)
+Gαβ +c/ebp(MIP-1α)
None
+ Gα
+ Gαβ
AC
A
BCX
AB
A
1 2 3 4 5 6 7 8 1 2 3


b
None
+Gα
+Gα +FPα short
+Gα +c/ebp(MIP-1α)
+Gαβ
+Gαβ +FPα short
+Gαβ +c/ebp(MIP-1α)
None
Gα alone
AC
A
BCX
A
1 2 3 4 5 6 7 1 2

These results suggest that GABPα seems to be forming a complex with a C/EBP factor, most probably C/EBPβ. This complex formation with C/EBPβ may be responsible for the unexpected results obtained with regards to GABP-induced activation of the *defensin* promoter in HeLa cells. Recent studies on the eosinophilic EOS47 promoter have shown strong co-operative transactivation between Ets factors (primarily Ets-1) and C/EBP factors (C/EBPα and C/EBPβ) (239). Co-operative binding between such factors was also observed when adjacent ets and c/ebp sites are present. GABPα is an Ets family member in the same family subgroup as Ets-1 and Ets-2 (390). It is not impossible to conceive therefore that a similar interaction may occur if GABPα is added in excess of its GABPβ partner. The adjacent ets and c/ebp sites within FPα would allow such a complex to bind DNA.

14.3 A C/EBP-containing complex binds to FPα in the presence of GABPβ

Further studies into the nature of these complexes indicated a complex picture as can be seen in the EMSA in Fig.57b. Again, addition of GABPα (lane2) results in formation of the band A and also enhances the intensity of a lower mobility band due to the formation of complex AC. Prior to addition of GABPα, this band may be due to the presence of GABPαβ bound to FPα as this would have the same mobility- band AB(see lane 3- right hand panel- Fig.57a). In other words, this band may represent both AC and AB complexes. A C/EBP-binding oligonucleotide from the M-*CSF Receptor* (M-CSFR-lane 4) promoter once again reduces the intensity of this band

(probably by competing away complex AC). In order to distinguish better between the components of the complexes formed, a short version of the FPα oligonucleotide was also used as competitor. This included the core GGAA ets site and a few adjacent nucleotides on each side but excluded the c/ebp and aml sites downstream of this site. It reduced band AC intensity to some extent and also band A intensity, showing that GABPα was still binding to this shorter oligonucleotide. However, when this competitor was added to GABPαβ with HeLa extract (lane5) the result was a clear reduction in the intensity of band A associated with a very marked increase in the binding of the low mobility complex marked as band BCX (lane 6). This suggests that band BCX does not include GABPα (despite being formed upon the addition of GABPβ). On the other hand, competition with a C/EBP-binding oligonucleotide (*M-CSFR* promoter) resulted in a reduced intensity of this band BCX and increased binding of GABPα alone.

These results therefore suggest that GABPβ is involved in a protein-DNA complex with a C/EBP factor (most probably C/EBPβ) and probably without GABPα (due to increased binding after GABPα binding is competed away). This GABPβ-C/EBPβ-containing complex is prevented from binding to FPα due to the binding of GABPα to the FPα ets site.

These EMSA results form the basis of a hypothesis which might explain most of the functional results of the GABP transfections in HeLa cells (and also possibly in NB4 cells). This hypothesis would depend on competition between GABPα and GABPβ for C/EBPβ forming a strongly active complex in the former case and an inactive complex in the latter. This is elaborated further in the discussion (section 16.8).

14.4 GABP sub-units bind to the minimal *defensin* promoter as complexes different from GABPαβ

The -67/+15 *defensin* promoter fragment was labelled with [γ^{32}P]-dATP and HeLa nuclear proteins were added to it together with purified GABP proteins (Fig.58). GABPα protein without HeLa extract binds to DNA weakly alone (band A) or as the GABPαβ complex (band AB) when GABPβ is added. Both GABP sub-units bind DNA more strongly, however, as different-sized bands together with HeLa extract (Fig.58a, lanes 4 and 5). The DNA-protein complex formed when GABPα alone (AX) is added (Fig.58a, lane 4) is of higher mobility (and therefore probably smaller in size) than that formed when purified GABPαβ is added (lane 2 - longer exposure).

Since the AX complex has a greater mobility than the AB complex, it is unlikely to be the AC complex seen on FPα. Alternativley, AX may indeed be the FPα AC complex and the AB band seen on this longer promoter fragment may be the GABP$\alpha_2\beta_2$ tetramer not the dimer explaining its lower mobility. GABPβ added together with HeLa extract (lane5) also forms a DNA-bound complex (BX) which is distinct from that formed between GABPβ and GABPα. This is particularly interesting since GABPβ is incapable of binding DNA alone and is thought to require interaction with GABPα. Here it appears to be binding to DNA by means of an interaction with an alternative DNA-binding partner. Another alternative is that the band formed does not include GABPβ at all but is caused by DNA-binding proteins which were previously bound to GABPα and which could not interact with the DNA and which have now been released by GABPβ interacting with GABPα in their stead. Addition of both GABP sub-units (lane 6) results in formation of complexes similar to those seen with addition of each separate sub-unit as well as one (band ABX) of lesser mobility that the complex

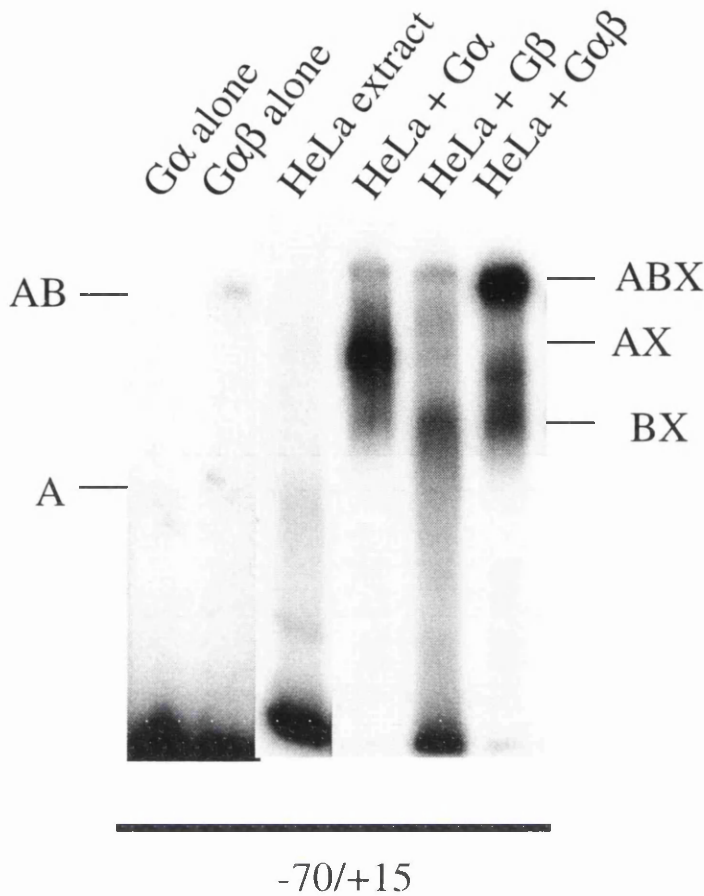


Fig.58 GABPα and β form complexes of the –70/+15 defensin promoter fragment with HeLa , distinct from those formed by GABPαβ.

GABPα (Gα), GABPβ (Gβ) - 25 ng of each, and HeLa extract (5μg) are incubated in various combinations with radiolabelled defensin upstream sequences (–70/+15) in the presence of buffer 3. The complexes formed are separated by electrophoresis through a 4.5 % polyacrylamide gel. Band A (which is barely visible) and AB represent the complexes formed by the purified proteins alone. Complexes AX, BX and ABX indicate the interaction of other HeLa factors (X) with the different GABP sub-units.

formed with purified GABP$\alpha\beta$. This would suggest that another factor is binding together with GABP$\alpha\beta$.

The exact components of the bands AX, BX and ABX are difficult to guess partly because they may include a number of factors bound to different sites along the length of the minimal promoter element. The intensity of these new formed complexes in comparison to those formed by HeLa extract alone or by the purified proteins does however suggest that they involve co-operative binding of sorts between GABP sub-units and HeLa cell factors.

Discussion

Chapter 15 : Critiques of *in vitro* and transient transfection experiments

The various experimental systems made use of in this study allow clear and reproducible gathering of experimental data. Using this data one can extrapolate probable DNA-protein and protein-protein interactions and formulate hypotheses regarding the likely behaviour of the gene being studied *in vivo*. However, each of these methods is clearly artificial.

15.1 NB4 and HeLa cells as model systems for studying *defensin* gene regulation

I have used these two cell lines to carry out these studies due to their particular characteristics. The NB4 cell line had been previously shown to express *defensin* (96). Despite not being as widely used as HL60, it has been well characterised both as regards its genetic make-up and morphological/stage of differentiation characteristics and also regarding its *in vitro* differentiation potential. It was much more easily transfected than HL60, (in my experience) and thus a better choice for studying promoter activity during the differentiation process. Like any model cell line it differs to some extent from the primary myeloid tissue (see section 1.3.3).

Down-regulation of *defensin* gene expression with terminal differentiation, diverged from the complete down-regulation seen with primary myeloid cells (see section 1.1.6). There appeared a lack of concordance between the morphological appearance of the cells and related level of expression of the gene. For this reason, as well as other logistical reasons relating to the transfection of differentiating cells, I concentrated my studies on defensin up-regulation in early differentiation. Numerous controls were performed regarding the transfection of reporters into NB4 cells to ensure that the effects seen were specific to the test promoter and that they were not general effects due to cell changes with differentiation.

HeLa cells have been widely used as a cell line with a low level background of myeloid transcription factors. They have also been previously used when studying primary granule protein genes (321).

That transfections into both the HeLa cells and NB4 cells, produced similar (if unexpected) results with GABPα expression vectors was important. The similarity lends support to this being a real effect and not an in vitro culture-induced side effect on one clone of cells of a particular cell line. This concordance of results between different cell lines suggested that there was no change in our clones of NB4 and particularly HeLa cells from those used in previously published experiments, but that other differences in the experimental system might play a role in the unexpected results. The reporter system used was identified as one probable cause.

15.2 *In vitro* footprinting assay

This experimental system was very useful in studying the *defensin* promoter though results require caution in interpretation. The titration of the amount of nuclear

stages of differentiation. The temptation to use large amounts of nuclear extract, thus obtaining more clear cut images of footprints should be tempered against the relevance of the footprints thus obtained to the *in vivo* situation. This point is clearly illustrated in our studies with differing amounts of nuclear proteins. Whilst more obvious footprints are obtained using larger amounts of nuclear proteins (200μg), it is clear that differences between the different extracts are more clearly seen when using smaller amounts of extract (40μg). Therefore a titration using differing amounts of nuclear proteins (preferably more extensive than my limited one) should be performed and the results assessed in order to best extrapolate to the *in vivo* situation.

15.3 Electrophoretic mobility shift assays (EMSAs)

In order that EMSAs are informative, the experimental environement often needs be modified so as to optimise the particular interactions being studied. This may require changes to a reaction (or running) buffer, as is often done for reactions with purified GABP proteins since these proteins need a low salt concentration in order to bind effectively to DNA in this artificial test environment (292;322). Similar modifications may also be essential to optimise particular antibody-antigen interactions. Different buffers or different acrylamide gel concentrations may be required to best observe the effects of competitor sequences (or other reaction additives) with a longer labelled probe sequence.

The variability in the resultant band patterns even when using the same probe sequence is not an indication of erroneous experimentation but an understandable side-effect of creating differing artificial environments to try and enhance various

interactions. Within a single experiment, such variables as probe, nuclear extract type, separating gel density and reaction buffer should be kept constant. This allows significant differences between separate reactions (seen in different lanes) to be identified (as long as adequate controls are taken into consideration such as the use of a pre-immune serum control for polyclonal antibodies and a non-specific monoclonal for monoclonal antibodies).

For this reason, modifications of this experimental system (EMSA), so long as they result in experiments that are reproducable in themselves, need not be a cause for concern. Results obtained following such changes to the experimental system may be considered relevant since modification of an artificial system does not make this system any more foreign to the actual test (or target) gene or protein *in vivo.* It is the *in vivo* situation that all experiments are attempting to understand and upholding consistency of experimental methods for consistency's sake is not any kind of scientific virtue (in my opinion).

Whilst indicating which factors within a particular nuclear extract can bind a particular site in isolation in a set of *in vitro* circumstances, EMSAs do not distinguish particularly well as to which of these sites are actually occupied *in vivo*. Some indication can be obtained, however, by the relative strength of such binding. C/EBPε appears to bind to FPβ much more strongly than it does to FPα or FPγ. However, due to the limited DNA template, transcription factors which would normally bind at another site in the promoter, may interact with other factors thereby attaching themselves to the test sequence in a manner not replicated *in vivo.*

15.3 Transient transfections for studying promoter activity

Tethering a reporter gene onto promoters allows one to assay relatively easily the activity of the promoter in question, despite the fact that is only an indirect measure. This indirect quantification may introduce errors in the sense that the actual quantity measured (such as in this case the expression of the growth hormone molecule itself) may be effected by the procedures tested such as differentiation or the expression of some transactivating factor. This is particularly the case when the reporter gene used is not a protein which is foreign to the cell species tested (e.g. luciferase or chloramphenicol acetyl transferase in mammalian cells). Despite such potential pitfalls, reporter assays allow promoter mutations or co-expression of transactivators to be tested with relative ease unlike the much more direct, yet more difficult, nuclear run-on assays of promoter function.

With both the experimental systems, however, the promoter is not being studied in its natural environment. It is not embedded in chromatin nor is it next to the sequences that it normally lies adjacent to. It is becoming more apparent that chromatin plays a crucial role in the regulation of transcription (103).

In fact, the *defensin* promoter sequence tested in transient transfection reporter gene studies, showed considerable up-regulation upon differentiation but this did not equate with the marked increase in mRNA abundance seen on Northern blots (especially for longer promoter fragments). This suggests (assuming that changes in transcription are the major variable effecting *defensin* mRNA accumulation) that other factors possibly related to increased accessibility of the chromatin-embedded promoter, or due to distant enhancer sequences, play a major role.

15.4 Reporter gene systems and promoter analysis

As mentioned before, the fact that GABPα produced a totally unexpected effect in two separate cell lines led me to believe that the difference was not in my particular grown clone of the cell lines in question, but in something common to both experimental setups, namely the reporter system. The initial test performed to assess this, confirmed these suspicions. The *cd18* promoter appeared to be up regulated by GABPα more strongly than by GABPαβ when cloned into the growth hormone reporter gene vector. In fact GABPαβ together did not activate the promoter at all whilst GABPβ alone was actually inhibitory. These results differed clearly from those obtained with the same promoter cloned into a luciferase reporter vector (322).

In luciferase based reporters (which have been almost exclusively been used to date in studying GABP), GABPα and β only ever transactivate a promoter when co-transfected together. This activation is then enhanced tremendously by other transcription factors such as C/EBPs and Myb on the *neutrophil elastase* promoter (321). However different luciferase based reporter vectors have been used to study GABP-induced transactivation (317;391), always with the same results, suggesting that any differences were not due to vector sequences peculiar to one particular luciferase reporter construct but due to something common amongst them, possibly the luciferase gene itself. When scanned using a promoter-identifying neural network algorithm (392) the luciferase cDNA sequence, was found to contain four very strong hypothetical TATAA box-dependant promoter sequences (each stronger by this reckoning than either the *defensin* or the *cd18* promoter). The growth hormone cDNA does not contain such strong hypothetical promoter sequences when analysed in the same way. Based on this finding and on our preliminary results, it is possible that in promoter studies using a

luciferase reporter gene all TATAA-binding protein (TBP) is bound to the strong TATAA sites within the luciferase cDNA thereby competing it away from the test promoter TATAA box. If this is indeed so, any transactivators bound to associated enhancer elements would stabilise TBP binding to the alternative promoter start sites and would be unable to activate the basically non-functional test promoter unless a TATAA-independent alternative initiator were also present. A similar phenomenon has been seen to occur where TBP is drawn away from promoter sites to UV or otherwise damaged DNA which binds it more strongly resulting in repression of transcription(393). GABPαβ can initiate transcription independently of a TATAA box (325) and would therefore be able to substitute for the loss of TBP-dependent initiation. However either GABP sub-unit alone would be unable to do this. Therefore promoter activity would only be detected in the presence of an alternative initiator sequence to the TATAA box and the presence of its requisite binding factors GABPαβ. With luciferase-based vectors, this would be the case both where GABP is possibly important for transcription initiation *in vivo* (like the TATAA-less *cd18* promoter) as well as in promoters where TATAA box-dependent transcription is normally the case (like the primary granule protein promoters – as is neutrophil elastase). Thus despite the possible interaction of GABPα with a co-operative transactivating partner, no reporter activity would be detected due to the lack of a GABPαβ initiator.

Studies using non-luciferase reporter genes (CAT) have also shown co-operative transactivation by the two GABP sub-units whilst neither were active alone(329). This shows that other parameters apart from the reporter gene system are important in the massive GABPα-induced transactivation seen on the defensin promoter.

Chapter 16 : Analysis of defensin promoter binding and activity

16.1 Factors binding to FPα

EMSAs performed using labelled FPα as a probe identified a number of factors that bound this site. These included GABPα, which clearly bound to the DNA in more than one complex in undifferentiated NB4 cells (see Fig. 25a). As the described complexes shifted by anti-GABPα antibody are not influenced by GABPβ antibody, it seems that in undifferentiated NB4 cells, GABPα seems to bind DNA in the absence of its usual partner and possibly together with other factors (lower mobility band). In differentiated cells, GABPβ-containing complexes (probably in combination with GABPα) are seen to bind FPα. The buffer system optimised for the function of anti-GABP antibodies resulted in the appearence of rather distinct bands with the FPα sequence. Other buffers showed more diffuse bands, but more clearly, an increased binding of granulocytic NB4 extract to FPα. Such increased binding is reminiscent of that seen by Ma *et al* (54)with almost the same probe sequence and using nuclear extract from another myeloid cell (HL60) induced with retinoic acid. This increased binding in HL60 cells was due to a phosphorylation-dependent Ets transcription factor. Our experiments suggest that this factor may well be GABP (probably as GABPαβ).

Mutation of the ets site shows the great functional importance of the Ets factor/s binding here, again confirming the report by Ma *et al* (54).

AML family members are also capable of binding to FPα DNA according to EMSA. The binding site for this factor is further downstream from the GABP-binding ets site, adjacent to a c/ebp binding site, and protein binding to this site is seen to increase (by DNAse1 footprinting) with NB4 cell differentiation (be it monocytic or granulocytic). This correlates with the increase in AML-1 known to occur upon differentiation of myeloid cells (338;354).

C/EBPε is also seen to bind to FPα by EMSA analysis though this is only detected in undifferentiated cells. This is contrary to the effect seen by footprinting where increased protection of the potential c/ebp site is seen in differentiated as opposed to undifferentiated NB4 cells. This may indicate that, C/EBPε binding to FPα as detected by EMSA would not occur on a longer promoter fragement in undifferentiated cells due to binding at other preferred sites. In differentiated cells, C/EBPε may bind strongly to FPα in a manner inaccessible to antibodies (as may be the case on FPβ mutated at the myb site). C/EBPα has been shown to bind to a rat *defensin* promoter fragment extending till just upstream of FPα as have been the AML family members AML-1B, AML-2 and AML-3(53). However co-operativity between C/EBPα and AML-1 on this promoter deletion mutant was not recognised. CBFα(AML) and Ets factors which both appear to bind FPα, are known to bind co-operatively with C/EBPs (see section 3.4.2). Whilst C/EBPε in particular has not been shown to interact either with Ets or AML factors, it appears to be involved in low mobility (high molecular weight complexes) on FPα in which it interacts with other DNA-binding factors by means of its N-terminal domain (see Fig. 32). Both with FPα- and FPγ-bound complexes, disruption of C/EBPε binding by anti-N-terminal antibody is not associated with appearance of higher mobility (lower molecular weight) complexes. This indicates that the factors C/EBPε interacts with, are unable to bind FPα, in its absence suggesting

that DNA binding requires a co-operative interaction. The C/EBPε C-terminal domain appears to be tightly bound to other proteins since addition of anti-C terminal antibody does not have any effect of these complexes suggesting it cannot access the protein. (The lack of any effect may be related to a relative weakness of this antibody-antigen interaction considering that the antibody to the C-terminal is raised against the rat homologue of C/EBPε, CRP1).

Further evidence of C/EBP binding to FPα came from competition EMSAs which indicate that numerous proteins can bind to this sequence (as was also suggested by computer analysis) but that these proteins bind to the DNA depending on an affinity hierarchy. Fig. 59 is a cartoon indicating possible binding interactions on FPα as seen by EMSA. In undifferentiated cells the rather non-descript or hazy bands seen, signify a state of continuous equilibrium with one or more complexes binding to, and dissociating from, the DNA. Competing away either PU.1 (*cd11b* oligonucleotide) or GABP (FPα short) results in the more intense appearance of a C/EBP band (as identified by antibody and competing oligonucleotides) as well as enhanced binding of complexes marked as "H". These rather small factors/complexes (shown as high mobility bands) may possibly be Ets factors of a sort since they are competed away by the neutrophil elastase Ets-binding site. They are unlikely to be GABPα since their binding is also increased (and not competed away) by the FPα-short oligonucleotide competitor which binds GABPα (see Fig. 35b). Factor "H" binding is also increased upon competing away C/EBPε.

Ets factors may inhibit C/EBPε binding in two ways, either by competing for binding to an overlapping ets-c/ebp composite site in FPα, or by interacting with the C/EBP factor's DNA-binding domain (as they are depicted doing in Fig.59) and preventing its binding to FPα. Such DNA-domain interactions are known to occur

Fig.59 Model for FPα competition EMSAs.

(a) FPα competition in the presence of undifferentiated NB4 cell nuclear extract. The dark black labeled line indicates a particular oligonucleotide of DNA. Reversible arrows indicate unstable binding whilst dark heavy arrows indicate the direction of the process during competition. DNA sequences to the sides of these heavy arrows indicate competitors and the complexes on them indicate the hypothetical factors and/or complexes they bind. Transcription factors are indicated by different shapes as described. GABPα (green triangle), PU.1 (pink triangle), Ets (indiscriminate – pink/green hatched triangle),factors H from EMSA –Fig 36 (yellow ellipse), GABPβ (complex shape with blue rectangle signifying the GABPα-interacting domain and the white ellipse the transactivation domain), C/EBPε (complex shape with a dark blue ellipse signifying the basic DNA-binding domain, a black zigzag, signifying the leucine zipper and two small ellipses signifying the repression domain - white ellipse - and the transactivation domain, the red ellipse). When these two small ellipses in C/EBPε are opposed as one larger ellipse, C/EBPε is taken to be inactive; when they are separated, it is derepressed.

(b) Oligonucleotide competition of differentiated proteins binding to FPα.

Model is based on the same figures as described for Fig. 59(a). AML is signified by a yellow hexagon whilst a large red arrow marked TA signifies a protein complex capable of transactivation in that it can interact with the basal transcriptional machinery.

Gα
PU.1
Cε
Gα
?H
Gβ
PU.1
or
Gβ
Gα

cd11b ets site(pu.1)

defensin FPα(short)

a

FPα(Undiff.)

ETS
or
Cε
H

FPα(Undiff.)

H
Cε

M-CSFR c/ebp site

FPα(Undiff.)

Cε
AML
PU.1
Gβ
Gα
PU.1
Cε

cd11b ets site(pu.1)

c/ebp site

FPα(Diff.)

b

TA
TA
Gα
Cε
AML
Gβ
Gα

FPα(Diff.)

FPα(Diff.)

(394), and in certain cases can inhibit transcription factor-DNA binding. Whilst interactions between the DNA-binding domain of C/EBP and Ets factors have been described(239), their role (if any) in inhibiting C/EBP binding has not been investigated to my knowledge. Removal of both Ets and C/EBP factors results in loss of all binding except for complex "H" indicating that most factors bound to this site do so co-operatively with Ets and/or C/EBP factors.

EMSAs using differentiated NB4 extract show, a similar pattern of competitive binding of different factors with the main difference being that GABPβ (probably with GABPα) now appears to bind to FPα in the absence of C/EBPs (modelled in Fig. 59b). The high mobility complex "H"-factors are not included here for simplicity and because they seem to bind DNA less avidly in differentiated cell extract. When GABPα is released from the GABPαβ complex by GABPβ antibody, the DNA binding of these factors increases (Fig. 37b). The released GABPα may displace the "H" factors from complexes with other transcription factors (possibly by substituting them) thereby allowing them to bind DNA.

16.2 Factors binding to FP β

EMSA analysis showed C/EBPε to be bound strongly to FPβ when using differentiated extract. GABPα is also shown to bind FPβ within NB4 granulocytic nuclear extract though it is not capable of binding the site as a purified protein (not shown). It is not clear whether GABPβ is bound together with GABPα to FPβ. The latter may be interacting with other proteins such as C/EBPs or AML in order to bind to this site. Competition EMSAs where FPβ mutants compete away FPγ-bound proteins,

suggest that AML as well as C/EBP proteins are bound to the site. Computer analysis of the sequence suggest that Myb may also bind to this site despite the fact that anti-Myb monoclonal antibodies did not shift any complexes (not shown). A myb-site mutated FPβ oligonucleotide is as poor a competitor of FPα-bound proteins (probably C/EBP) as the c/ebp site mutant(Fig.23a). This suggests that C/EBP binding onto FPβ is co-operative with Myb (or at least with a protein binding the myb site) in undifferentiated NB4 cells. Whilst co-operative binding between Myb and C/EBP proteins has not been shown, this has not been well investigated in the case of C/EBPε, where this may be more likely, due to the monomeric nature of this protein. Both the lack of a supershift by anti-C/EBPε antibodies and the reduced C/EBPε-dependent transactivation following myb site mutation support this hypothesis of co-operative binding.

However FPβ mutated at the myb site is an even better competitor of FPβ protein binding by granulocytic NB4 extract than wild type FPβ oligonucleotide, unlike the c/ebp-site mutant FPβ. This suggests that C/EBPε may still be bound to FPβ in the absence the myb site and that the protein interactions are more complex than can easily be resolved by such *in vitro* analysis. It is possible that in the absence of Myb, C/EBP may be able to bind strongly to FPβ by co-operative interactions with other factors present only in differentiated nuclear extracts. Such FPβ-bound C/EBP, though more strongly attached to the DNA, may be inaccessible to the antibodies and functionally inactive.

C/EBPs and Myb interact with each other through their respective DNA-binding domains (235) and they both interact functionally (and physically in the case of C/EBPs) with the same part of the AML-1 runt-homology domain (295;352). This triangle of interactions therefore suggests that C/EBP factors can only interact with

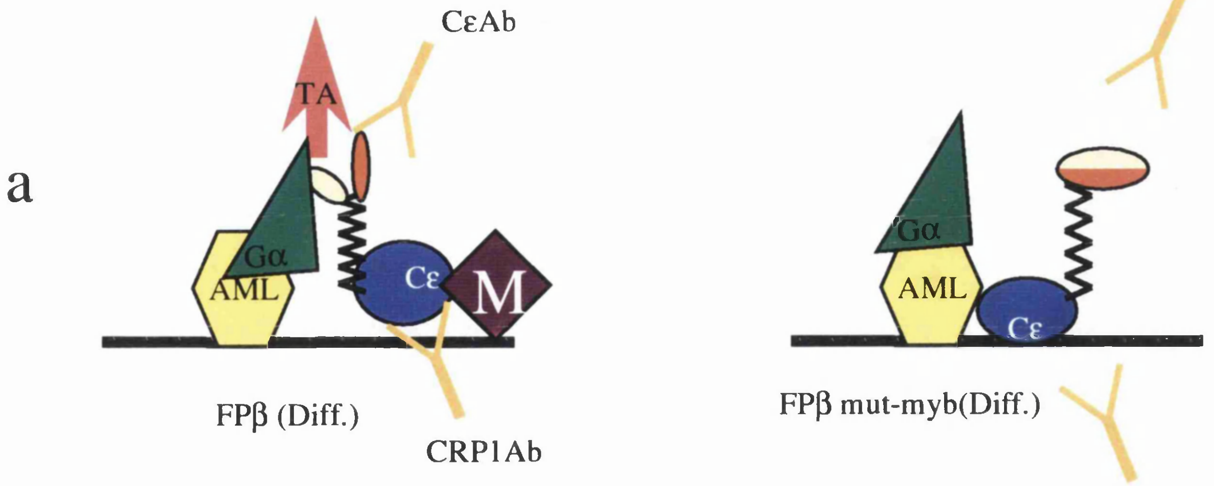
Myb or AML at any one time. This may explain a situation where a C/EBP may interact more strongly to one factor (AML) resulting in strong co-operative binding yet such interaction may prevent synergism with Myb. A cartoon showing hypothetical transcription factor interactions on wild type and myb site-mutated FPβ oligonucleotide with granulocytic NB4 extract is shown (Fig.60a).

16.3 Proteins binding to FPγ

The main change in protein binding seen on FPγ with granulocytic differentiation is that of increased PU.1 binding (seen as the high mobility doublet usually marked as bands 3 and 4). Whilst AML proteins, C/EBPε and even GABP are all shown to possibly bind sequences around this footprint, the relatively weak binding seen with footprinting suggests that most transcription factors would bind to other sites in the presence of the whole promoter sequence. This suggests that many of the multi-protein complexes formed on the labelled FPγ probe sequence are in fact artefacts of the experimental system, in the sense that they are seen due to the isolation of this particular binding site from other binding sites.

PU.1 on the other hand appears to bind most strongly to this site (and possibly to a sequence close to the TATAA box (54)) within the promoter and such binding is probably responsible for the hypersite seen upon differentiation. Fig. 60b shows a model giving a possible explanation for the band complexes seen with GABP antibodies (Fig. 31c). Once again it is unlikely to represent the *in vivo* situation, as most if not all GABP would be probably bound to FPα which much more closely resembles the GABP consensus-binding site.

Fig.60 Model of DNA-protein interactions on FPβ and FPγ. (a) C/EBPε binding to FPβ and to FPβ-mut-myb. Model based on similar representative symbols as Fig 63. Additionally, Myb is represented by a purple diamond shape and antibodies are represented by orange 'Y'-shaped figures. The model attempts to show how enhanced DNA-binding can correlate with reduced transactivation activity therefore attempting to bring together disparate EMSA and functional data.
(b) Effects of GABPα antibodies on FPγ EMSAs. Model is based on similar representative symbols as before. The trapezium N-133 signifies the PU.1 proteolytic product shown in EMSAs as band 4 (which is not necessarily the same protein as the in vitro produced N-133 at all). Heavy arrows indicate either addition of GABPα antibody or differentiation under the influence of the inducer all-*trans* retinoic acid (ATRA).



b

Cε
PU.1
Gα
FPγ (Undiff.)

ATRA

Gβ
AML
Cε
Gα
and
PU.1
Main complex (band 2) (band 3 complex)

FPγ (Diff.)

GABPα Ab

GABPα Ab

GαAb

Gβ
Gα

Cε PU.

GαAb

and

N-133
Gα
(band 4 complex) supershifted complex

AML
Cε
PU.1

In Fig.60b, the complex shown binding FPγ by undifferentiated nuclear extract may represent the complex seen as one of the lower mobility bands such as bands 1 and/or 2 in EMSAs (see Fig. 31c). Band 1 may (as has been mentioned) also involve the binding of some non-specific DNA-binding protein, which may be competed off by various different sequences (see Fig.30). C/EBPε is pictured as binding to the DNA via protein interactions through its N-terminal domain and with its C-terminal domain obstructed by protein- protein interactions as is suggested by antibody studies. Addition of a GABPα antibody would supershift a GABPα-DNA complex but would also disrupt interactions with the main FPγ-bound complex. C/EBPε and PU.1 interacting through their DNA-binding and other domains would be unable to bind DNA. PU.1 lysis products lacking domains important for C/EBP interaction such as the N-terminal domain (240) other than the ETS domain, would be able to bind DNA left vacant by the disruption of the GABPα-containing complex. This would explain the appearance of band 4 in Fig. 31c.

With differentiated extract, the PU.1 binds directly to DNA in preference of GABP as a result of changes in the abundance of other transcription factors with differentiation, (which explain the reduced supershifted band formed by GABPα antibody).

The model suggests that GABPα interacts with C/EBPε's N-terminal domain. This interaction is only hypothetical and may in fact be between GABPα and any of several other bound/unbound transcription factors/factor complexes. Anti-GABPα antibody would inhibit such interactions thereby releasing the GABP co-operative partners for interactions with other Ets factors. The Ets factor PU.1 fills this new role in the absence of the antibody-bound GABPα and in so doing does not bind DNA directly any longer, resulting in a reduction of the band 3 in Fig.31c-differentiated extract panel.

16.4 Competition and/or co-operation between different sites in the same promoter

Competition EMSAs also show that the binding of one transcription factor to its particular site (such as PU.1 to FPγ), result in enhanced binding of other transcription factors to different sites (such as C/EBPε to FPα) (see section 9.3.2). Similarly removal of C/EBPε resulted in enhanced binding of GABP$\alpha\beta$ complex to FPα.

Therefore two important points emerge; firstly that different transcription factors can compete for the same binding site and secondly, that particular factors, bind to certain preferred sites within the promoter at limiting concentrations. Taking these points into consideration, the presence, number and distribution of binding sites for each particular factor within the upstream sequence of a gene may play a crucial role in optimising binding of that, and other factors, and subsequent promoter activation. It is a well recognised feature of myeloid promoters that binding sites for different factors seem to follow a particular pattern(185).

The binding of transcription factors in co-operative manner to DNA has been well investigated but the inhibitory effect of such factors on each other's binding is less well recognised. In the interesting study on the EOS47 eosinophil promoter(239), the authors described the co-operative binding of C/EBPα and Ets-1 to a DNA sequence containing binding sites for both transcription factors. The same study clearly shows (though this is not described) that in the absence of a binding site for C/EBP, addition of this factor (DNA-binding domain only) in fact reduces the binding of Ets-1 DNA-binding domain. This correlates with my studies which suggest that unbound

transcription factors (such as PU.1 in relation to FPα), can prevent the binding of other factors (such as C/EBPε) to their binding sites.

This understanding allows the results of mutation analysis to be taken in a new light. Mutation may reduce promoter activity, not only as a result of a positively transactivating factor being unable to bind to DNA. This factor as a result of its non-adherence may inhibit the binding of other transactivating factors. A case in point may be pu.1 binding site mutation analysis. In undifferentiated NB4 cells the -140/+15 promoter fragment activated the reporter gene as strongly (at least) as the -240/+15 fragment. However, mutating the PU.1-binding site in either of 2 ways, results in a drop in reporter activity. One way of looking at this is that PU.1, when bound to the DNA, is unable to interact with other factors like C/EBPε and inhibit their binding. Alternatively, the inability of PU.1 to bind FPγ might result in this mutated site binding other factors more strongly (see Fig.29). This may result in such factors being drawn away from sites where they may be transcriptionally active (yet less strongly bound) to a site where they may be inactive.

16.5 C/EBPε and GABP regulate *defensin* expression in NB4 cells

GABPα and C/EBPε had a clear transactivatory effect in NB4 cells. This indicates that the factors are in limiting concentration in these cells and increases in their abundance can result in upregulation of promoter activity (see section 12.3). This may be due to their binding to more sites along the length promoter or to more effective competition with other transcription factors for binding to the same sites. It may also be

due to the factor being present in sufficient abundance to bind DNA over and above interactions with any inhibitory factors or unbound protein complexes.

C/EBPε is known to increase during early myeloid differentiation, so such co-transfection may mimic the differentiation process as regards this factor. On the other hand GABPα does not seem to increase with differentiation so that the GABP transfection-dependent up-regulation is likely to be due to another mechanism.

16.6 GABP-dependent *defensin* transactivation is enhanced by NB4 cell myeloid differentiation

We have shown by Western blotting analysis that no great change in the abundance of the GABP sub-unit proteins occurs during early granulocytic differentiation of NB4 cells. According to functional co-transfections in myeloid cells, however, transfected GABPβ synergises with GABPα in differentiated NB4 cells but does not appear to do so in undifferentiated ones. EMSA suggests that GABPβ is not bound to FPα DNA in undifferentiated cells yet is, in differentiated cells. Purified GABPα and GABPβ proteins made in *E.coli*, on the other hand, did bind as a GABP heteromer when added to a FPα probe sequence in the presence of undifferentiated NB4 nuclear extract. This suggests that it is probably the abundance of these two proteins in relation to other myeloid transcription factors within the extract, which govern their interaction with each other and their subsequent binding to the *defensin* promoter.

Whether or not GABPα and β interact, they do not co-operate in undifferentiated NB4 cells as co-expression of GABPβ with GABPα does not enhance

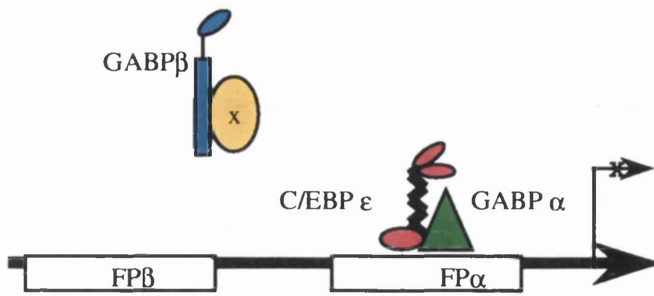
the activity of the latter. The synergism that occurs upon differentiation is unlikely to be due to any small change in the relative abundance of the two sub-units. The synergism may possibly be due to post-transcriptional modifications (though these must be small since there is no clear changes in molecular weight visible on western). Such modifications may enhance the capability of the two sub-units to interact, or reduce their interaction with alternative partners.

One possible mechanism for such post-translational modification is phosphorylation. Changes in GABP phosphorylation are well recognised and are dependent on the MAP and other kinase cascades (329;395). MAP kinase activity is stimulated during early G-CSF-induced granulocytic differentiation on a myeloid cell line (396), a process similar to ATRA-induced granulocytic differentiation of NB4 cells. Increased phosphorylation-dependant binding of an Ets factor to the *defensin* FPα site (which may well be GABPαβ) has been shown to occur upon ATRA-induced HL60 differentiation (54).

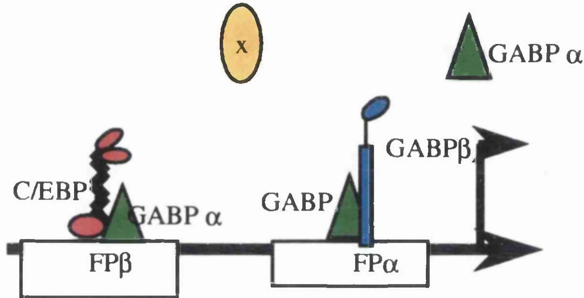
Another explanation for the increased co-operation of the GABPαβ sub-units with differentiation may be due to changes in the abundance of protein partners interacting with each sub-unit. These alternative partners may be down regulated with differentiation. Other factors which may in turn interact with these alternative GABP partners may also be up regulated with differentiation, thus releasing GABPα and β to interact with each other.

If the two GABP sub-units are not interacting with each other in undifferentiated NB4 cells, co-transfected GABPα might redress the ratios of interacting partners (modelled in Fig.61). The excess GABPα would be able to interact with GABPβ as well as with its alternate partner/s resulting in formation of an active GABPαβ transcription factor complex and subsequent promoter transactivation. Adding more

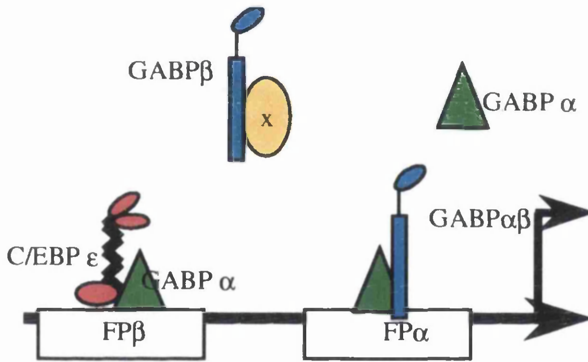
Fig.61 Model of GABP-dependent transactivation in NB4 cells.
Model based on the same descriptive symbols as Fig. 59. The elliptical factor X, signifies an unknown factor which may be interacting with GABPβ, thus preventing its interaction with GABPα and with the DNA.



-240/+15 defensin

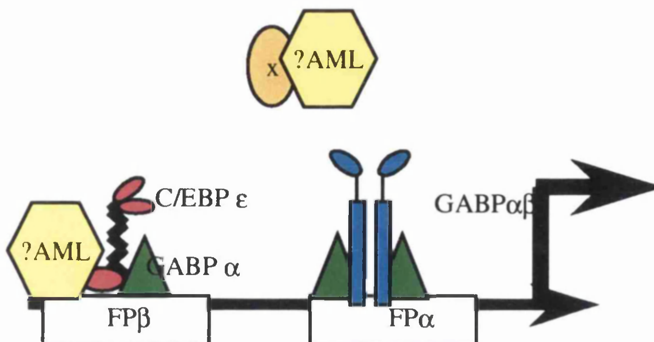


-240/+15 defensin
+ GABP α



-240/+15 defensin
+ GABP αβ

Differentiation



-240/+15 defensin
+ GABP αβ
(differentiated cells)

GABPβ would have no increased effect, since it would once again bind its alternate partner (shown as X). After differentiation, both GABPβ and α alternate partners are shown interacting primarily with other factors upregulated by differentiation. Excess GABPα can interact with GABPβ and its own alternate partner causing strong transactivation as in undifferentiated cells. However, addition of GABPβ would allow all the GABPα and β to interact and further transactivate the promoter.

16.7 Myb and C/EBPε synergistically trans-activate the *defensin* promoter

This study has shown clear-cut evidence of a strong synergism between c-Myb and C/EBPε on defensin promoter activation in HeLa cells. In other sudies, however, co-expression of Myb enhanced transactivation by wild type or repression domain-deleted C/EBPε to the same extent (224). This suggested that Myb was not synergising with C/EBPε by overcoming the effect of the latter's repression domain. It also suggests that C/EBPε de-repression may require interaction with another factor. Ets factors have previously been shown to interact with the N-terminal domain of C/EBPs (240) where the activation and repression domains lie (153;224). They may be responsible for the de-repression of C/EBPε.

The mechanism of Myb-C/EBPε synergism seen on the *defensin* promoter is presently unclear. Co-operative binding to DNA was never shown between any C/EBP family member and Myb despite their interaction. FPβ competition of FPα-bound undifferentiated NB4 proteins does indicate that both c/ebp and myb sites are required

for effective competition suggesting the possibility of co-operative DNA binding. The relative inability of C/EBPε to homodimerise (370) or heterodimerise with most C/EBP family members (153) would lend itself to a hypothesis of co-operative binding with Myb.

In the presence of differentiated NB4 extract, however, possibly due to an increase in AML and for other co-operative binding partners of C/EBP, the myb site does not seem essential for strong FPβ binding unlike the c/ebp and aml sites. Despite this, the presence or absence of myb sites seems to change C/EBPε binding since antibody-C/EBP interactions on FPβ are lost with mutation of the myb site. In NB4 cells, mutation of either the adjacent FPβ c/ebp or myb sites strongly inhibit promoter activity.

On the other hand co-operative C/EBPε and Myb transactivation of the promoter in HeLa cells was independent of the FPβ c/ebp site suggesting that co-operation between the two factors does not depend on an overlapping DNA binding site. Such co-operativity can occur with sites for the two factors being separated by at least 10 nucleotides (FPα1) in HeLa cells which argues against co-operative DNA binding as a mechanism of functional co-operation. This may occur, however, by means of a bridging interaction of CBP/p300 with the two factors, as has been hypothesised (209). Thus whilst compelling, the evidence for co-operative binding is incomplete at best.

In NB4 cells on the other hand, the Myb bound at FPβ cannot co-operate with C/EBPε bound at alternate sites like FPα, α1 or γ to transactivate the promoter. This difference may be due to other transcription factors present in NB4 cells (like AML-1) which may interact with the C/EBP through the same domain with which it interacts with Myb, thereby competitively inhibiting its interaction with the latter. The lack of

such C/EBP-interacting factors in HeLa cells may allow C/EBPε bound at more distant sites to interact and co-operate with Myb despite the distance.

16.8 GABP proteins may interact physically and functionally with alternative partners

The HeLa cell experiments show a marked transactivation of the HNP-3 defensin promoter by GABPα in excess of GABPβ. These results suggest that GABPα is interacting functionally, if not also physically with other partner protein/s. EMSAs show bands upon the addition of GABPα, the formation of which is inhibited by the addition of C/EBP binding oligonucleotides as well as by the addition of anti-C/EBPβ antibodies. These data suggest that GABPα may be binding to the FPα DNA sequence with C/EBPβ.

Recent studies have shown strong co-operative transactivation by Ets factors (namely Ets-1) together with C/EBPα or β in the presence of adjacent binding sites for the two factors on the EOS47 promoter (239). Interactions were shown to be dependent on the ETS domain of the Ets factors and the basic zipper domain of the C/EBP. Therefore in the right circumstances, it is feasible that GABPα may interact with C/EBPβ, which is present in HeLa cells. Addition of GABPβ inhibits the marked transactivation produced by GABPα upon co-transfection. This inhibition is not so marked at all when GABPγ is co-transfected. Since GABPβ and GABPγ are similarly capable of binding with the ETS domain of GABPα, the reason for this difference must relate to the GABPβ homodimerisation/transactivation domain.

Addition of purified GABPβ in EMSA experiments did not result in the formation of the characteristic GABPαβ sized band, but a lower mobility complex (named BCX). This band was also reduced in intensity by two different recognised C/EBP binding oligonucleotides suggesting the possibility that GABPβ was interacting with C/EBPβ (this being the C/EBP found in HeLa). GABPα may itself form part of this complex (i.e. a GABPα-C/EBPβ-GABPβ complex). Competing away GABPα binding, by a FPα core ets site oligonucleotide, however, enhanced the binding of this complex, suggesting that the above is unlikely. An interesting hypothesis for GABPβ-inhibition of GABPα-induced transactivation can therefore be formulated. By competing for the same C/EBPβ interactive partner, GABPβ prevents GABPα from forming an active complex resulting in an inactive one instead. The inactive GABPβ-C/EBPβ-X complex is probably primarily found in solution (or bound to a site other than FPα) since GABPα binds to the FPα site in preference to the BCX complex.

If there is indeed an interaction between GABPβ and C/EBPβ, this is most likely to occur via the leucine zipper regions of both proteins though no such interaction has yet been shown. GABPβ's leucine zipper is required for GABPβ dimer formation(324). GABPγ, on the other hand, lacks this dimerisation interface. This may therefore explain why GABPγ does not inhibit GABPα-induced transactivation so much since it would not be able to interact with C/EBPβ as would GABPβ.

Within NB4 cells, interaction of GABPα with C/EBPε may be responsible for the marked transactivation of the *defensin* promoter seen upon co-transfecting GABPα, though we do not have any evidence to support this idea. GABPα may alternatively be interacting with some other factor like AML-1, another well-known co-operative partner for Ets factors.

In these myeloid cells, co-transfected GABPβ does not reduce the GABPα-induced activity. This may be for various reasons, the most important of which is that GABPa-induced transactivation may be occurring by a different mechanism as suggested by the models in Chapter 17. Differences in the C/EBP activators found in the different cell lines and their interactions with the two GABP sub-units may also be of importance

16.9 Incongruities between exogenous and endogenous promoter activation

Following these studies, T Jamieson, a colleague, transfected HeLa cells with different activators that transactivated the *defensin* promoter strongly in transient transfections. None of the transfected factors, including those known to strongly transactivate the promoter such as GABPα, or the combination of C/EBPε and Myb, increased endogenous *defensin* gene expression (data not shown). *Defensin* mRNA was not detected in northern blots of any of the transfected HeLa cell populations.

The reasons for this difference are unclear, though endogenous gene up regulation has been shown vary rarely in heterologous cells (186). Since the transcription factor environment for both reporter gene or endogenous promoters is the same (HeLa cells), the main reason for these differences probably lies in their different chromatin structure. Reporter gene constructs provide an easily accessible promoter whilst the promoter of the endogenous gene in a non-expressing cell may be buried deep within the heterochromatin and not accessible to transactivators.

Heterologous defensin expression was attempted in epithelial HeLa cells by co-transfection of transcription factors activating the promoter . A rare study where myeloid gene expression in heterologous cells was achieved, (186), used other mesenchymal cells like erythroid cells or fibroblasts as the test cell line . It is possible that the greater ontological diversity of the epithelial HeLa cell line from the tissue in which the gene is normally expressed may influence the extent to which that gene is inhibited by chromatin. HeLa cell have also been shown to be a poor background for C/EBP-induced transactivation on occasion (233).

These major differences between transient transfection promoter studies, and endogenous gene expression emphasise the limitations of this experimental system in studying gene regulation *in vivo.* Greater understanding of how chromatin structure influences transcription (as they so clearly do in transgenic animal experiments) may help translate transient transfection results onto endogenous genes in the future.

Chapter 17 : Models of *defensin* promoter interactions

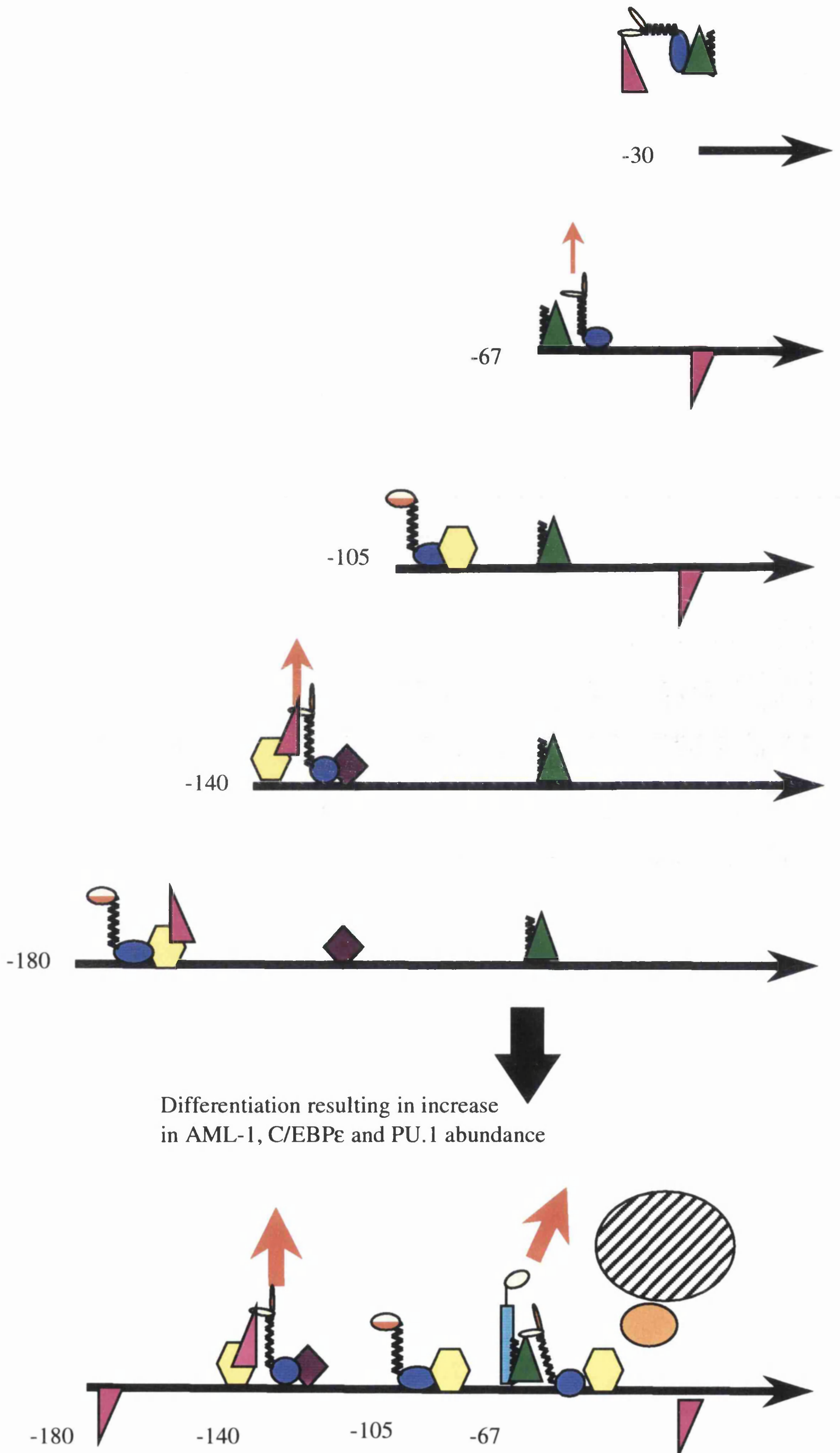
The knowledge gathered from *in vitro* experiments regarding the interactions between transcription factors on the *defensin* promoter can be used to construct models regarding the results of transient transfection experiments.

17.1 Model of transcription factor interactions on promoter deletion mutants transfected into NB4 cells

Fig. 62 shows a cartoon depicting how transcription factors may interact on the different deletion mutants of the *defensin* promoter transfected into NB4 cells. On the shortest inactive (-30/+15) promoter fragment, there are few if any binding sites for transcription factors. TBP binding to the TATAA box is not in itself enough to tether the RNA polymerase complex to the promoter and initiate transcription. The binding of factors to sites present in this short deletion mutant, such as the functionally important (possible c/ebp) site identified in HL60 studies (-30/-20), and the PU.1-binding site at position (-23/-17), may be limited, due to interactions with other unbound factors which prevent their interaction with the DNA(as indicated).

Inclusion of FPα (-67/+15) increases the number of transcription factor binding sites and may indirectly enhance binding to other sites as suggested by competition EMSA experiments (see Figs. 36,37). This may result in GABPα (green triangle) binding to DNA at one site (FPα) and PU.1 (pink triangle) binding at another site (-18) with C/EBPε bound next to either of the Ets factor. Interaction between the Ets factor and the adjacently bound C/EBP's DNA-binding domain and possibly its repression

Fig.62 Model of transcription factor binding to defensin promoter deletion mutants. The model is based on the same representative transcription factor symbols as in Fig. 59 The orange ellipse signifies the TATAA-binding protein TBP whilst the black and white hatched large ellipse signifies the rest of the basal transcriptional machinery. The model expounds the hypothesis that including new DNA sequences can alter the distribution of transcription factor binding on the exposed DNA. This may enhance or inhibit the formation of active complexes by these different sites within the same promoter competing for particular factors. Differentiation is shown to enhance the expression of numerous transcription factors such as to allow occupation of both inactive and active DNA binding sites.


-30
-67
-105
-140
-180
Differentiation resulting in increase
in AML-1, C/EBPε and PU.1 abundance
-180
-140
-105
-67

domain may result in de-repression of its activation domain. This would allow the DNA bound complex to interact with the transcription machinery, recruiting the polymerase and enhancing promoter activation.

Inclusion of sequences upstream of FPα till nucleotide -105 includes strong c/ebp-aml binding sites. This may result in C/EBPε being drawn away from the Ets factors with which it was co-operating to another site where it is inactive. AML-1 is known to bind DNA and transactivate promoters co-operatively with C/EBPα (see section 3.6.4). Such co-operative interaction, however, may also require Ets and/or Myb proteins(352;397). C/EBP bound to the FPα1 c/ebp-aml site would not be able to interact with adjacent DNA-bound Myb or Ets factors within the context of the *defensin* -105/+15 promoter.

Inclusion of the FPβ incorporates a Myb-binding site. The effect of this is modelled here with C/EBPε being held to the DNA by means of a co-operative interaction with Myb (purple diamond). Such co-operative interaction may be indirectly using such bridging factors as CBP/p300. Meanwhile, an adjacent Ets factor (shown here binding to the DNA through a co-operation with AML-1) interacts with the C/EBP repressor domain (white ellipse) releasing the activation domain (red ellipse) to interact with the polymerase complex and recruit it to the *defensin* promoter. Here the Ets factor shown binding FPβ (in a co-operative manner with AML-1) is PU.1 whilst EMSAs showed GABPα to bind this site. However, FPα (the strong GABPα-binding site) is present (in the –140/+15 promoter fragment) whilst FPγ (the strong PU.1 binding site) is absent. Therefore in the context of this promoter fragment it is possible that PU.1 rather than GABPα will be bound to this site.

Inclusion of the sequences up till -180 incorporates FPγ, which introduces another strong C/EBP- and AML-1-binding site thereby luring C/EBP from the active

complex formed on FPβ. The added ets site may also sequester ets factors from FPβ (such as PU.1) also resulting in an inactive complex. Now there are two binding sites (FPα1 and FPγ) which could bind C/EBPε in an inactive mode (away from Myb) as opposed to only one potential active site (FPβ). Inclusion of the sequences up to nucleotide –240, include another potential myb-c/ebp combined site restoring the balance of active to inactive C/EBPε binding sites (not shown in model).

Upon differentiation, the amount of C/EBP and AML-1 is increased such that enough is present so as to occupy both the inactive and the active sites, and the increased PU.1 binds strongly to FPγ thus possibly preventing C/EBP binding to this site. The increased PU.1 may also interact with C/EBPε in place of GABPα thereby enhancing GABPβ co-operation with GABPα whereupon they can also bind the polymerase and transactivate the promoter. This combination of changes may explain the increased promoter activity due to far better recruitment of the basal transcription machinery.

Whilst the cartoon model depicts the promoter here as a straight line, recent studies have shown that multiple transcription factors, bound to DNA, bend the DNA around the polymerase complex thus enhancing the interaction between each factor and the basal machinery and stabilising the initiation complex (353). DNA-bending proteins like LEF-1 play an important role in such complex formation. By mutating the binding site for such a protein, promoter activity may be markedly reduced despite it not having much transactivating potential in itself.

The FPα ets site-bound protein/s (including possibly GABPα) may play a similar role apart from having an important role in transactivation. A clear hypersite, which may be associated with DNA bending, is seen just upstream to this ets site and Ets factors have been shown to bend DNA to a considerable degree. Mutation of this

ets site results in the total loss of activity of the promoter even when the factors activating the promoter bind DNA totally independent of this site (such as C/EBPε and Myb in HeLa cells). In view of the fact that this FPα ets site appears essential for activation in all circumstances, it is possible that factors bound here bend the promoter DNA. This would allow improved interaction of factors bound along the length of the promoter to the TATAA box-bound basal machinery thus stabilising the complex and better activating the promoter.

17.2 Interactions on the *defensin* promoter in HeLa cells

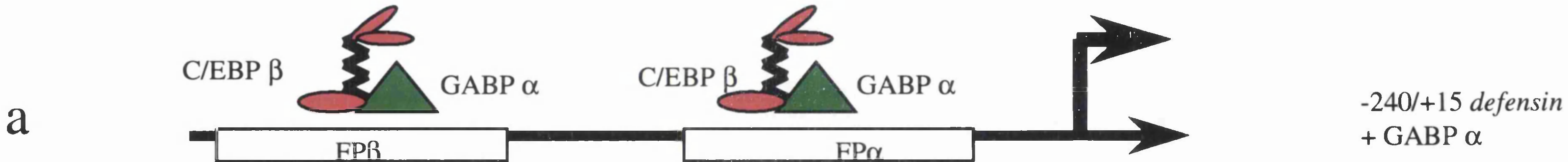
As in myeloid NB4 cells, within the HeLa cellular environment, interactions between different sites may play an important role in the promoter activity of different constructs co-expressed with different activators. Mutation of the c/ebp site in FPβ is not at all deleterious to promoter activity induced by C/EBPε and Myb in HeLa cells. In NB4 cells, both c/ebp and myb FPβ sites are crucial for activity. A likely explanation is that C/EBPε not bound adjacent to Myb in NB4 cells, is likely to interact strongly with other factors such as AML family proteins bound to other sites along the promoter. C/EBPs physically interact with Myb through the same domain with which it interacts with AML(295). Both C/EBP and Myb functionally interact with the same domain protein sequence of the AML-1 factor. Therefore in NB4 cells, stearic constraints may prevent more than two of these proteins from interacting optimally with each other and the separation of the DNA binding sites for these different factors might dictate which interactions predominate. In HeLa cells, this three-way competition for interaction does not occur since AML-1 is not expressed to any great extent. Therefore Myb bound to FPβ could interact with C/EBP bound to FPα or more likely to FPα1.

In the case of GABPα-induced transactivation, mutation of the FPα ets site clearly effects GABPα binding. Mutation of the FPβ c/ebp site (which also reduces GABPα-induced transactivation) may reduce the potential binding sites for a GABPα-C/EBP complex (FPβ was shown by antibodies to be able to bind both C/EBPε and also GABPα in NB4 cells). Alternatively, C/EBPε, being unable to bind FPβ might interact with and inhibit GABP binding to FPα. Mutation of the core ets site in FPγ also reduces another site where C/EBP and GABPα may bind adjacently. Similarly, removal of the sequences upstream of FPα reduces the potential binding sites for such a complex. All site-specific of deletion promoter mutants may therefore reduce GABPα-induced activity.

Fig. 63 shows a cartoon outlining a likely model of the co-operativity between the GABPαβ heteromer and various other factors such as AML-1/CBFβ, PU.1 or CHOP-10. The top panel shows the –140/+15 promoter fragment schematically in the presence of excess GABPα with GABPα-C/EBPβ active complexes forming on the promoter. The middle panel shows the situation with GABPβ added resulting in C/EBPβ bound to GABPβ together with another protein marked as X.

Addition of CHOP-10 would result in C/EBPβ-CHOP-10 dimers forming, these being incapable of binding DNA. GABPα and β having had their alternate partner removed are free to interact and bind to the vacant FPα ets site thus transactivating the promoter. PU.1 if added in to a shorter promoter fragment (-67/+15), whilst interacting with C/EBPβ would probably bind to the FPα ets-c/ebp site in a co-operative manner. This would prevent the reformed GABPαβ complex from efficiently binding DNA and transactivating. On the other hand, within the longer promoter sequence, this newly formed Ets-C/EBP complex may bind to FPβ (or FPγ) allowing the GABP heteromer

Fig. 63 Model showing interactions between the GABPα and β sub-units and other factors. C/EBPβ is pictured as was C/EBPε but all in red, otherwise the same symbols as in Fig 59 are used.
(a)Shows the hypothetical situation with GABPα expression resulting in strong activation by co-operation with C/EBPβ. This co-operative complex may bind both FPα and FPβ.
(b)Shows the situation with GABPβ complexing together with C/EBPβ and possibly other factors (here indicated as X). This complex will be unable to bind strongly to DNA in the presence of GABPα as well as possibly being inherently inactive. The lowermost panel shows the situation when PU.1, AML-1 or CHOP-10 are added to GABPαβ.


a
C/EBP β
GABP α
C/EBP β
GABP α
FPβ
FPα
-240/+15 *defensin*
+ GABP α
b
X
FPβ
FPα
-240/+15 *defensin*
+ GABP αβ
PU.1
AML-1
CHOP
CHOP-C/EBP β
AML-C/EBPβ
PU.1-C/EBP β
GABPαβ
FPβ
FPα
-240/+15 *defensin*
+ GABP αβ + PU.1 or
AML-1 or CHOP-10

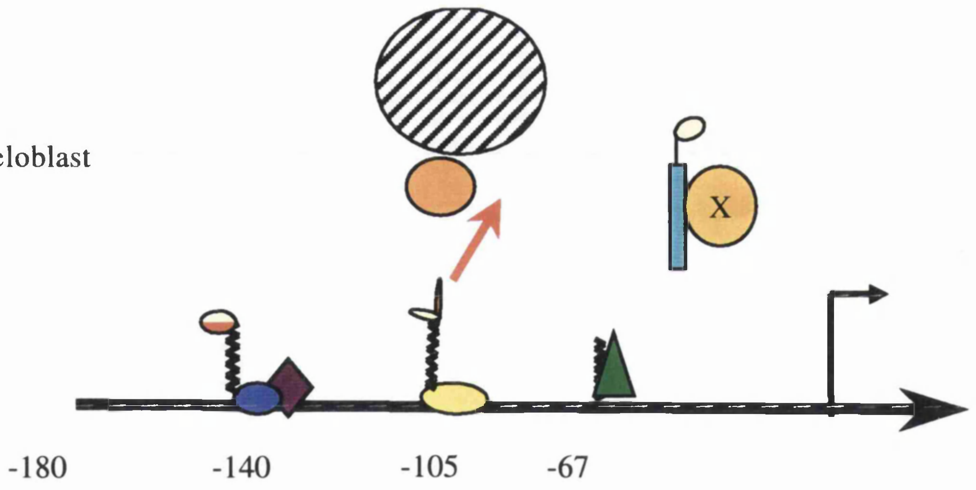
to transactivate the promoter through FPα. AML-1 may co-operate with GABPαβ in a very similar fashion.

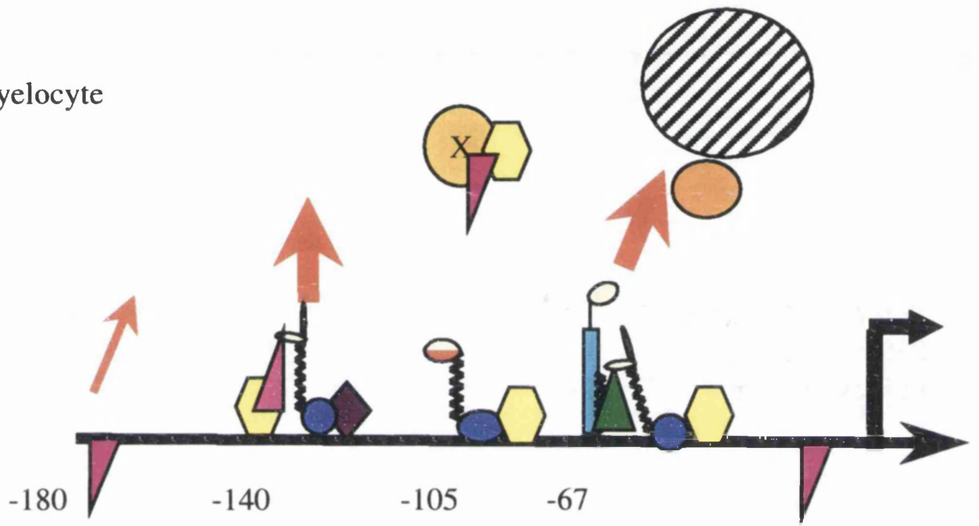
17.3 Stage-specific expression of *defensin* as a function of changes in the abundance of various transcription factors

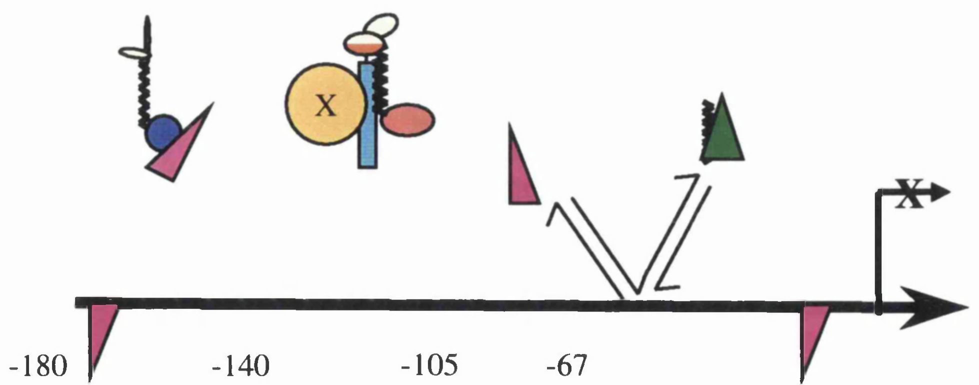
All this data, together with previously published information, makes it possible to present a model of how transcription factor interplay on the *defensin* promoter may cause the increased expression of the gene during early myeloid differentiation and the subsequent down-regulation later on (Fig.64). Prior to *defensin* expression during myeloid commitment, the chromatin around this gene locus may be modified to allow for improved access of the genomic DNA by transcription factors. In previous cell divisions during myeloid differentiation, the FPα CpGGAA site is demethylated, thereby allowing the binding of GABPα to this site. The binding of this factor may allow low level *defensin* gene expression, possibly as a result of DNA bending within the promoter resulting in co-operative interactions between other bound factors.

Early myeloid cells (myeloblasts/promyelocytes) express good levels of c-Myb and also C/EBPα together with some C/EBPε. These factors may co-operate with Myb at FPβ and transactivate the promoter. The C/EBPs may also bind to FPγ together with PU.1 and to FPα together with GABPα though the role of such complexes in transactivation is uncertain. Other factors important in transactivation, like GABPβ, may be complexed to alternate protein partners preventing them from playing an active role in transactivation. Following myeloid differentiation into myelocytes, an increase in C/EBPε will occur, together with a decrease in C/EBPα and an increase in AML-1 and possibly in PU.1.

Fig. 64 Model to show the up regulation and subsequent down regulation of the defensin promoter with differentiation. The same representative symbols are used as in previous figures. C/EBPα and β are represented by yellow and red versions of the same C/EBP symbol.


Myeloblast
X
-180
-140
-105
-67


Early Myelocyte
X
-180
-140
-105
-67


Metamyelocyte
X
-180
-140
-105
-67

The change in the relative abundance of the C/EBP factors should result in improved synergism with Myb on FPβ. The increase in AML will probably allow enhanced co-operative Ets binding to FPβ resulting in a possible active AML-Ets-C/EBPε-Myb complex. Increased PU.1 will bind to FPγ, where it itself may aid in transactivation and in so doing may displace the C/EBP and AML factors to other sites where they may be more active.

GABPα can better interact with GABPβ now that the alternate partners of both sub-units (including possibly the C/EBPs) can interact with other factors such as PU.1 and AML-1 instead. The GABPαβ dimer can now therefore further transactivate the promoter through FPα. C/EBP proteins, at this stage, are abundant enough so as to bind to c/ebp-aml sites on FPγ and α1 (inactive) as well as to FPβ (active) together with AML and possibly with PU.1.

Upon further differentiation, C/EBPε is down regulated (according to some studies (374) but remains strongly expressed according to others (386)). Myb (also see Fig. 38) and AML-1 are also down-regulated with differentiation, whilst PU.1 is further up regulated as is C/EBPβ(21). GABPα and β are probably relatively unaffected.

These changes can in themselves indicate a probable mechanism of *defensin* mRNA down-regulation. Switching C/EBPε for C/EBPβ results in a family member, which does not appear capable of co-operating strongly with c-Myb on myeloid promoters(218). Even if C/EBPε is still expressed, the down regulation of Myb will result in the co-operative transactivation potential of this transcription factor partnership being lost. In the absence of protein partners like c-Myb and AML-1, C/EBPβ (and possibly C/EBPε) will begin to interact with alternative factors. Through its interaction with GABPβ, C/EBPβ will split the active GABPαβ complex forming a situation similar to that in HeLa cells with GABPα bound alone to an

inactive promoter. The abundant PU.1 present at this stage can compete (either alone or co-operatively with C/EBPβ) for binding to the FPα core ets site. This may result in displacement of GABPα which may result eventually in methylation of FPα and permanent repression of the gene.

Most of the factors mentioned here which appear to be responsible for *defensin* expression are also present in lymphoid cells (255). However, the presence of a methylated cytosine nucleotide in the FPα core ets site, detected in lymphoid lineage leukemia cells (A. Philips/T Jamieson unpublished results) probably inhibits GABPα binding and promoter transactivation, thereby limiting *defensin* expression to the myeloid lineage.

Chapter 18 : General conclusions and future work prospects

A number of very interesting findings have resulted from these studies on the *defensin* promoter, not the least of which is the possibility of novel transactivating and interacting partners for the GABP factors sub-units. However, a considerable amount of work can yet be done.

18.1 Completing the picture - EMSAs and further functional analysis of the minimal promoter

Many of the EMSAs performed gave rise to very interesting results. As a result of following up certain such results, analysis of other sequences was not as thorough, occasionally, as it could have been. Adding AML antibodies to EMSA studies on all footprints would have been most interesting, as would have been western analysis to document changes in the abundance of this factor specifically duriong the granulocytic maturation of the NB4 cells. More uniform and detailed analysis of each footprint by competition EMSA with both undifferentiated and differentiated NB4 extracts is also important.

Any number of transient transfection experiments could be proposed to help complete the picture, yet two stand out as rather essential. Firstly, co-transfecting C/EBPβ (and the different C/EBPs) together with GABPα into NB4 cells should help discover whether this combination of factors is indeed responsible for the massive up-regulation in promoter activity seen upon GABPα transfection into HeLa cells. NB4 would make an ideal background, as they do not express C/EBPs other than C/EBPε.

Similarly, co-expressing C/EBPε and GABPα in HeLa cells without Myb might help explain the mechanism of GABPα-dependent up-regulation seen in NB4 cells.

The second important experiment would be to perform more extensive mutational analysis on the minimal promoter sequence in order to identify the sequences required for optimal promoter activity in differentiated NB4 cells. This minimal promoter whilst not very active in undifferentiated cells is just as active as the longer promoter fragment constructs in differentiated cells. Thus it is of interest to identify functionally important sequences other than the FPα ets site by mutagenesis. These may include the c/ebp and aml sites within FPα itself or the PU.1-binding site or the functionally important (potentially C/EBP-binding) site identified by Ma *et al* (54). Such transfection studies, due to the numerous factor binding-sites within a relatively short DNA sequence might provide valuable insights into how the myeloid factors on this core promoter interact in promoting transcription.

18.3 Understanding intra-promoter binding site competition and co-operativity

Few publications in the literature describe competition between different transcription factor binding sites within the same promoter (or adjacent promoters in the DNA) and the effects of such competition on transcription (398-400). More describe the competition of different factors for the same binding site within the promoter(401).

Competitive transcription factor binding to FPα was analysed as part of this study. Extending the same kind of studies to the other footprints would be very interesting. One can further extend these studies by assessing the effects of

oligonucleotide competitors on DNA-protein complex formation, when they are added in equimolar quantities (thereby mimicking the promoter template) and not at 50-200 fold the molar concentration used in these EMSAs.

Transfection studies with co-transfected decoy oligonucleotides representing particular transcription factor binding sites may also help elucidate factor-factor interactions on the promoter. Such co-transfectoins should mimic the effect of mutating the site if such mutation removes a positive transactivator from the promoter. However, if mutation relaeses a factor which when unbound to DNA inhibits the DNA-binding or activity of other factors, a decoy should not replicate this negative effect.

18.3 Further analysis on the luciferase and growth hormone reporter genes

There is a clear difference in the transactivation of promoters by GABP factors depending on whether the promoter in question is cloned into our growth hormone reporter construct or a luciferase based one. I have argued (based on computer analysis of sites within the reporter gene sequences) that this may be as a result of TATAA binding protein (TBP) being competed away from the test promoter TATAA box to a number of different spurious "promoter" sequences within the luciferase cDNA. A reduction in transcription has been shown to happen when TBP is competed away from the TATAA box and onto UV-damaged DNA(393). To understand whether such a mechanism was responsible for the reporter gene differences, mutating the TATAA-dependent promoter sequences within the luciferase cDNA without disrupting the functional luciferase molecule would be helpful. If this was achieved, according to the proposed hypothesis, GABPα (within a suitable cellular background) should

transactivate promoters in this modified luciferase construct just as strongly as in our growth hormone reporter construct. Adding excess TBP (and possibly other basic transcription factors) together with co-transfected GABPα, might also allow strong GABPα-dependent transactivation from the standard luciferase vector.

18.4 Could GABP sub-unit imbalances result in the abnormal gene transcription?

Assuming that the GABPα effects we have discovered are not artefacts of our experimental system, are they ever of any significance *in vivo*? As described previously, GABPβ is ubiquitously expressed in most tissues together with GABPα despite the fact that they are expressed from two different chromosomes. Therefore the scenario we have shown in HeLa cells of a positive imbalance of GABPα as compared to GABPβ is probably unlikely to occur in normal circumstances.

Tumorigenesis however is well known to result in karyotypic instability especially in malignant tumours mutated in the p53 locus (402). Loss of chromosomes and amplification of others may occur resulting in such an imbalance. Here up-regulation of the *defensin* promoter is seen in an epithelial tissue not known to express this gene. Similarly, karyotypic anomalies leading to GABPα–β imbalances may result in the expression of genes in an inappropriate manner and this may further contribute to the malignant phenotype.

Possibly more significant is the fact that the human *gabpα* gene is located on the chromosome 21 (403), whose trisomy results in the condition known as Down's

Syndrome. This in itself produces a chromosomal imbalance between GABPα and GABPβ-carrying chromosomes, and could provide a clear mechanism for inequalities in the abundance of the two sub-units of this ubiquitous transcription factor. Abnormalities in gene expression secondary to such an imbalance especially if occurring at early stages of foetal life could easily play a role in the developmental abnormalities characteristic of this condition. These are fertile grounds for further research.

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