

**Transcriptional Control of the m4 Muscarinic Receptor Gene:
Mammalian And Yeast Models.**

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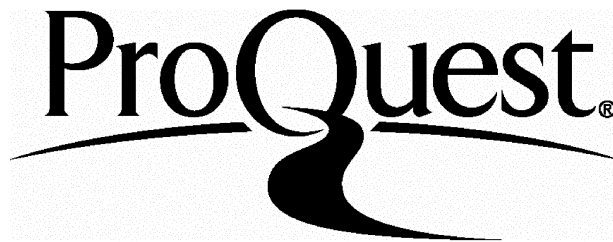
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Abstract.

The mammalian nervous system is comprised of approximately 10^{10} - 10^{11} neurones, each of which has a potentially unique repertoire of expressed genes. The m4 muscarinic acetylcholine receptor gene has a very restricted pattern of expression within the nervous system, being expressed mainly in the autonomic ganglia and telencephalon. Using transient transfection assays in mammalian cell lines I show that the m4 gene is under the control of a constitutively active core-promoter which is selectively repressed in m4 non-expressing cells. This repression is mediated at least in part by the zinc finger repressor REST. Further, in the fibroblast cell line 3T3, I show that the m4 gene is expressed via two distinct promoters which are differentially regulated by REST.

In order to dissect the molecular mechanisms that mediate REST repression I have expressed REST in yeast and shown it to be a potent repressor of the yeast GAL1 promoter. Further, characterisation of those regions of REST that are important for repression in yeast map to those regions that mediate repression in mammalian cells. This suggests that REST acts via mechanisms and molecules that are conserved through evolution between yeast and mammals.

To identify genes important in mediating REST repression, I expressed REST in the absence of the yeast global repressors SSN6 and SIN3. Deletion of SSN6 had no effect on REST mediated repression while as deletion of SIN3 resulted in complete loss of repression by REST. These experiments show that the conserved gene SIN3 is essential for REST mediated repression in yeast and suggest that SIN3 may act as a co-repressor for REST in mammalian cells. The implications of REST repressing via SIN3 and future directions based in these findings are discussed.

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CONTENTS.

List Of Figures.	5
List Of Abbreviations	7
Chapter 1: Introduction.	10
Chapter 2: Materials and Methods	41
Chapter 3: Transient Transfection Analysis of m4 Promoter Deletion Constructs.	53
Chapter 4: Isolation and Characterisation of DNA Binding Proteins.	79
Chapter 5: Analysis of REST Repression of the GAL1 Promoter in Yeast.	101
Chapter 6: Discussion.	120
References.	139
Plasmid Maps.	161

LIST OF FIGURES.

Figures.

CHAPTER 3

1. Expression of luciferase reporter constructs in transient transfection of NG108, PC12, CHO and 3T3 cell lines. 57
2. Comparison of the RE1/NRSE sequences from the m4 and other neural specific genes where this region has been identified as being functionally important in gene regulation. 61
3. Expression of luciferase reporter constructs in transient transfection of NBOK1 and Neuro-2a cell lines. 68
4. Expression of luciferase reporter constructs in Neuro-2a cell lines in the presence of recombinant REST. 70
5. Analysis of 3T3 cells co-transfected with m4 dual promoter constructs and RE1 containing PCR products. 74

CHAPTER 4

6. Open Reading Frame and RE1 binding analysis of 2TB5-1. 85
7. 1-hybrid candidate HM21-1 is a fragment of mouse REST. 88
8. Analysis of REST binding to the m4 and NaII derived RE1 elements. 92
9. Western analysis of REST DNA Binding Domain expressed at high and low levels in yeast. 94

10. Repression of GAL1-HIS3 by REST in yeast. 106
11. Human and mouse REST are highly conserved within the N-terminal repression domain, DNA binding domain and lysine rich region. 108
12. Western analysis of HZ4 and HZ4D1-138 expression in the yeast strain FM242. 111
13. REST mediated repression in yeast is SSN6 independent. 114
14. SIN3 disruption in yeast abolishes REST mediated repression but leaves glucose repression intact. 117

LIST OF ABBREVIATIONS

Ab	Antibody
AD	Activation Domain
Amp	Ampicillin
AMP	Adenosine Monophosphate
AS-C	Acheate, Scute-Complex
ATP	Adenosine 5'-Triphosphate
Bcd	Bicoid
bHLH	basic Helix Loop Helix
CAT	Chloramphenicol Acetyl-Transferase
CBP	CREB Binding Protein
CMV	Cyto Megalo Virus
CNS	Central Nervous System
CREB	cyclic-AMP Response Element Bindin protein
CTD	C-terminal Domain (of RNAPII)
Da	Daughterless
DBD	DNA Binding Domain
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DomNeg	Dominant Negative (DN)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
Emc	Extramachrochaete
EMSA	Electrophoretic Mobility Shift Assay
FTZ	fushitirazu
GAL	Galactose
GCN	General Control of Nutrition
GPR	G-protein Coupled Receptor
H	Hairy
HAT	Histone Acetylase Transferase
Hb	Hunchback
HD	Histone De-Acetylase
HDAC	Histone De-Acetylase
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HMG	High Mobility Group
HTA	Histone 2A

HTB	Histone 2B
IME	Inducer of Meiosis
Kr	Kruppel
KRAB	Kruppel Associated Box
Kb	Kilobase
K _D	Dissociation Binding constant
Kd	Kilodalton
LB	Luria Burtoni (broth)
Leonov	LEu2 expressiON Vector
m4	Muscarinic Acetylcholine Type4 Receptor
MAL	Maltose
MIG	Mediator of Inhibition by Glucose
Mr	Molecular Weight
NaII	Sodium Type II
N-CoA	Nuclear Receptor Co-Activator
N-CoR	Nuclear Receptor Co-Repressor
NRSE	Neural Restrictive Silencing Element
NRSF	Neural Restrictive Silencing Factor
OD	Optical Density
ONPG	<i>o</i> -nitrophenyl- β -D-galactopyranoside
ORF	Open Reading Frame
PAH	Paired Amphipathic Helix
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain reaction
PIPES	1,4-piperazinediethanesulfonic acid
Pol	Polymerase
RAR	Retinoic Acid Receptor
RE1	Repressor Element
REST	Restrictor of Expression of Sodium Type II
RNA	Ribonucleic Acid
RNAP	RNA Polymerase
SCG	Superior Cervical Ganglion
SDS	Sodium Dodecyl sulphate
SID	Sin3 Interaction Domain
SIN3	Switch Independent
SNF	Sucrose Non-Fermenter
SPT	Suppressor of Transposition
SRB	Suppressor of RNA polymerase B

SRC	Steroid Receptor Co-activator
SSN	Supressor of Sucrose Non-Fermenter
SUC	Sucrose
SWI	Switch
SV40	Simian Virus 40
TAF	TBP Associated Factor
TBP	TATA Binding Protein
TdT	Terminal deoxy-Transferase
Tet	Tetracycline
TF	Transcription Factor
TPR	Trico-Peptide Repeat
TSP-d	Transcription Start Point-downstream
TSP-u	Transcription Start Point-upstream
TSS	Transcription Start Site
UAS	Upstream Activation Sequence
UES	Upstream Essential Sequence
URS	Upstream Regulatory Sequence
2d-gal	2-Deoxy-Galactose

CHAPTER 1.

INTRODUCTION

CHAPTER 1.

INTRODUCTION.

Describing the establishment of cellular phenotype is one of the most fundamental questions in contemporary biology. Each cell type can be considered to express a unique, defined set of genes. Given that the mammalian nervous system alone contains as many as 10^{10} - 10^{11} neurones, each with a potentially unique repertoire of expressed genes, the problem of establishing regulatory networks to define this expression profile becomes astronomical. In order to define the mechanisms at play that produce this phenomenal diversity, we need a description of the gene as a functional unit and the factors that act on it to regulate its activity. Conceptually, a complete description of a gene will require defining i) the regulatory elements and the cognate transcription factors that act upon those elements, ii) defining the processes that occur post transcriptionally to give rise to mature mRNA transcript, and iii) defining the processes that occur post translationally which act to modify and modulate the activity of the newly folded protein. Identifying the genes that encode these functions will allow the analysis of the factors that regulate the regulators. The gradual illumination of the regulatory network in this manner will ultimately result in a full description of the mechanisms working at the molecular level that lead to the establishment of cellular phenotype which can be thought of as the sum of transcriptional and post-transcriptional processes within the cell.

My work reported here is an attempt to understand the processes that lead to the establishment of cellular phenotype and how these sum to produce a neuronal phenotype. I feel that these processes need to be

understood at the molecular level in order to provide a complete description. This invariably requires an understanding of gene regulation at the molecular level. There is a prevalent school of thought that assumes that genes can be treated as 'black boxes' which have inputs and outputs, the sum of which can give a satisfactory description of genetic control. In my mind, this is equivalent to treating the Periodic table as a satisfactory description of the elements. It is an understanding of the electron structures around nuclei that allows an appreciation of the Periodic table. Indeed many would argue that it is an understanding of Schrodinger equations that describe electron structure which is ultimately required to fully describe Mendeleev's arrangement of the elements.

In the same light, I feel that gene 'black boxes' as a means of describing the network of genetic interactions that give rise to neuronal phenotype is an inadequate description. To this end, in this report, I first précis what is currently understood about the molecular events that regulate individual genes. I then discuss the salient aspects of the model gene I use in this study, the type 4 muscarinic acetylcholine receptor gene. As is discussed later, the m4 gene is one of five muscarinic receptor genes. A neuronal gene which is a member of a family was chosen because ultimately, I hope to collate what is known about the regulation of the other family members and compare mechanisms. The fact that these genes have distinct but partially overlapping expression profiles should facilitate the transposition of what is known of individual promoters and regulatory mechanisms to what is known about when and where these genes are expressed. In other words it will help in bridging the chasm between the molecular

descriptions and the cellular/neuronal descriptions of the neuronal phenotype.

A MOLECULAR ANALYSIS.

Transcription starts when RNA polymerase binds to the promoter at the start of the gene. The promoter surrounds the first base pair that is transcribed into RNA, the transcription start point (see Lewin, 1997 for a review).

The story of promoter recognition, specificity and control of transcription began with the discovery of bacterial σ factors in *E. coli* (Travers and Burgess, 1969) and the demonstration that different batteries of cellular and viral genes were selectively transcribed by a cascade of σ factors in *Bacillus subtilis* (Haldenwang and Losick, 1980; Haldenwang *et al.*, 1981; Doi and Wang, 1986; Losick *et al.*, 1986; Losick and Stragier, 1992; Errington, 1993; Haldenwang, 1995).

Initially it was widely believed that similar mechanisms would be at play at eukaryotic promoters, presumably employing a very much larger array of σ like or σ equivalent factors to accommodate the larger number of genes. Although no direct homologue of σ factors exist in eukaryotic systems, as is discussed later, a number of factors found in the core complexes of eukaryotic promoters can be thought of as orthologous in that they act to impart promoter selectivity.

Any discussion about specificity of gene regulation is really one about how the transcription machinery is specifically directed to one particular promoter. The first eukaryotic factor isolated that could distinguish between promoters was Sp1 which Tjian and co-workers showed could direct transcription from the SV40 Early promoter but not

the Adenovirus Major Late promoter (Dyran and Tjian,1983a). Unlike the σ factors of prokaryotes however, this selectivity was attributed to specific binding of the Sp1 activity to a sequence element in the SV40 promoter not found in the Adenovirus promoter (Dyran and Tjian,1983b). This, and subsequent work carried out on yeast factors, suggested that eukaryotic promoters could be functionally divided into at least two domains: a proximal, or core region, which conferred specific basal levels of transcription and distal regions which act to up or down regulate the basal activity (Dyran and Yjian,1985; Zewel and Reinberg,1992).

A MOLECULAR ANALYSIS: The Proximal Promoter Complex

Proximal promoter regions tend to fall into 2 broad classes: those that contain a TATA sequence and those that do not possess such a recognisable motif. A glance at any selection of TATA containing promoters shows huge variation in the sequences flanking the TATA element. There is also a huge variability in basal promoter strength of promoters among cell types and also among different TATA containing promoters within a given cell type. This suggests that the protein apparatus sat upon the core promoter region is different in each case. These different activities are refined by distal sequences which recruit other proteins (activators and repressors) to produce the required level of transcription of each gene required by the cell.

What determines which promoters are modulated by which activators and repressors and what parameters govern the strength and selectivity of basal transcription? Inroads to these questions were made only very recently. The cloning and isolation of a factor that could bind the TATA element (TBP) opened the way to addressing the above questions (Cavallini *et al.*,1989; Hahn *et al.*,1989; Horikoshi *et al.*,1989;

Schmidt *et al.*,1989). TBP alone could support a low level of basal activity but failed to distinguish between one TATA containing promoter and another in terms of producing different specific transcriptional activities (Peter-Verrijzer *et al.*,1995). Further, TBP alone failed to allow activated transcription (Pugh and Tjian,1991). These observations implied the existence of other factors that possibly interacted with TBP to both modulate basal activity and allow activated transcription (Julien *et al.*,1990). Along with this, workers were hindered by the immense difficulty in purifying the TBP containing activity, TFIID, by chromatographic methods suggesting the existence of a multi-subunit complex.

The identification of other TBP Associated Factors (TAFs) allowed the exploration of the discrepancies seen between the properties of the TFIID holo-complex and TBP (Dymlacht *et al.*,1991), (Tanese *et al.*,1991). Addition of two TAFs to TBP, TAF_{II}250 (the largest TAF) and TAF_{II}150, caused a 10-20 fold increase of basal activity driven from a template containing an Adenovirus ML TATA element and another downstream element known as the initiator taken from the TdT gene. However, this ternary complex produced less activity from a template containing just the TATA element than TBP alone (Peter-Verrijzer *et al.*,1995). This suggested that the 2 TAFs were minimally required for defining promoter specific basal activities and that this was achieved through the selective destabilisation of TFIID when bound to an 'incorrect' promoter sequence. In many ways then, TAF_{II}150 and TAF_{II}250 act in an analogous manner to the bacterial σ factors in that they impart stability to a correctly assembled DNA-protein complex at the core promoter. Unlike σ factors however, addition of both TAFs enlarged the footprint of the DNA-bound protein complex down-stream of the transcription

start point implying that they bind DNA in a sequence discriminatory manner. This corroborates affinity labelling experiments. The most intriguing aspect of these experiments is that the stability of the promoter bound TBP-TAF_{II}150-TAF_{II}250 trimeric complex is very comparable to the TFIID holo-complex ($T_{1/2}$ =ca 25mins). This suggests that whatever TAFs are missing, they probably do not act to increase stability of the TBP-DNA interaction.

The trimeric complex above could support specific levels of basal activity and distinguish among promoters. However, it failed to respond to activators such as Sp1 where the TFIID holo-complex was able to be up regulated markedly under the same conditions. This suggested the presence of other factors that could act as mediators of the activation signal i.e. act as co-activators. TAF_{II}110 when added to TBP/TAF_{II}250 allows modest activation by Sp1 (Weinzierl *et al.*,1993). This suggests that activators such as Sp1 (so called 'glutamine rich') act through TAFs to mediate activation. TAF_{II}110 has a glutamine rich motif similar to that found in Sp1. In the latter case, Sp1 uses this glutamine rich domain to multimerise with other Sp1 molecules to produce synergistic effects at promoters (Hoey *et al.*,1993). However, it should be noted that although the C-terminal region of TBP is greater than 80% conserved across species, the N-terminal region is very divergent. In some cases, the N-terminal region contains a glutamine rich domain much like that of Sp1. It is conceivable then, that in these cases Sp1 can interact directly with TBP.

In summary, based on these mainly *in vitro* experiments and observations, eukaryotic promoters recruit TBP with associated TAFs to mediate basal activity and confer sensitivity to activators. It would appear that certain promoters recruit different TFIID complexes (i.e.

TBP with different constellations of TAFs) in order to establish the correct levels of basal and activated states (Pugh and Tjian,1991). Indeed, there is evidence that core complexes may define a particular set of activators that are permitted to act on a promoter and that this set may vary among cells for any given gene. So it appears that the core complex consists of TBP associated with various TAFs which act to modulate TBP promoter selectivity and activity and that this TFIID complex is associated with other complexes such as TFIIB, TFIIA and TFIIE, TFIIH, TFIIF all of which serve to produce a competent complex.

The question then is what of those promoters that lack recognisable TATA elements? Is the mechanism of transcription initiation at these promoters fundamentally different from that at TATA containing promoters? Intriguingly, TBP as part of TFIID is still required for transcription initiation at TATAless promoters (Pugh and Tjian,1991). However, TBP or TFIID isolated from TATA containing promoters is by itself unable to mediate specific basal transcription from a TATAless promoter. Rather, the addition of a 'tethering' factor is required to recruit TFIID to TATAless promoters. In the presence of this tethering factor, both basal and activated transcription is observed at TATAless templates. This suggests that the mechanisms of transcription initiation at TATA containing and TATAless promoters is fundamentally similar. In fact it may be the case that the tethering factor may be required simply to supply the extra binding energy to the DNA/TFIID complex and enhance complex stability in the absence of a TATA element. This scenario has an analogue in prokaryotes where the phage *lpRE* promoter lacks the required -35 sequence to recruit the *E.coli* RNAP complex. Instead, the transcriptional regulator *cII* binds to its recognition sequence at -35 and then presumably through protein-

protein interactions recruits the RNAP complex (Shimatake and Rosenberg,1981).

A MOLECULAR ANALYSIS: Activators.

Many activators work by binding to specific DNA sequences (enhancers) and 'touching' the core complex with an activation domain. This scheme is true for both prokaryotes and eukaryotes, however, unlike with prokaryotes, eukaryotic enhancer sequences can be located many kilobases up or downstream of the promoter in either orientation. How this class of activator performs its function upon binding its cognate element is thus a question of what events occur downstream of the activation domain making contact with the core transcriptional apparatus.

Eukaryotic activation domains tend to possess a number of shared structural themes. One structural theme is the lack of well defined tertiary structural motifs. The yeast activators GCN4 and GAL4 (Hope and Struhl,1986; (Ma and Ptashne,1987b) have activation domains consisting of a number of repeats. Serial deletion of this region results in less and less activation rather than an abrupt loss of activation (Wu *et al.*,1996). This suggests an unordered tertiary structure making contact with the core complex. Interestingly such a deletion series on GAL4 abolishes recognition and repression by gal80p (Leuther *et al.*,1993). Together, these observations point to an activation domain that although requires certain structural motifs to mediate inhibition by GAL80, does not require an ordered structure to mediate communication with the core complex. In higher eukaryotes, a similar theme is seen. The mammalian activator Sp1 has 4 repeats of a

glutamine rich domain and, indeed, potent artificial activation domains have been constructed containing homopolymeric repeats of glutamine residues (Gerber *et al.*,1994). Two 11 residue tandem repeats of an activation sub-domain of VP16 followed by 4 copies of a 5 residue motif were able to mediate efficient transcription in mammalian cells (Seipel *et al.*,1994). Two or three repeats of an 18 residue glutamine rich motif found in the homeodomain of Oct-2 is not only able to mediate activation when tethered to the Oct-2 DNA binding domain but can also mediate synergy with the adjacent POU domain just like the wild type 66 residue motif (Tanaka and Herr,1994; Tanaka *et al.*,1994). Again, as with the case of the GAL4/GAL80 interaction, the activation domain can perform functions other than just activation.

Activation domains have also been defined by the most common amino acids found within them. For example Sp1 is the prototypical glutamine rich type activator, VP16 is classed as acidic and Oct-2 is proline rich. Such nomenclature, though easy to see the origin of, is at best artifactual and at worst, misleading. For example, Sp1 can be shown to interact with TAF_{II}110. Mutation of the glutamine residues however has little effect on this interaction yet mutation of the bulky hydrophobic residues between the glutamine residues has severe detrimental effects on the Sp1/TAF_{II}110 interaction (Gill *et al.*,1991). However, this result should be seen in the light of other experiments where homopolymeric glutamine repeats have been fused to the GAL4 DNA binding domain and given potent activation in mammalian cells. Further, addition of leucine residues failed to increase activation. These apparent discrepancies may simply reflect the different experimental paradigms used to study activation: the glutamine mutation experiment looked at the *in vitro* affinity of Sp1 with TAF_{II}110 while the

homopolymeric repeat experiment analysed activation in transiently transfected cell lines. It is possible that there are multiple mechanisms at play, with the glutamine residues affecting one activation pathway and the hydrophobic residues acting via another (TAF_{II}110 dependent) pathway.

A MOLECULAR ANALYSIS: Mechanisms of activator action.

A list of what the targets are for various activators does not in itself constitute a satisfying description of the mechanisms of activator function at a promoter. A bewildering array of activator targets are known, some in the core complex as members of TFIID and TFIIA, others in the RNA PolII holocomplex and yet more in those complexes that modulate chromatin structure. It appears that activators act at all stages of transcription initiation from TFIID recruitment through to promoter clearance of the RNA polymerase.

The first step in transcription initiation is the recruitment of TFIID to the promoter (See Ptashne and Gann, 1997 and references therein for review). Many activators have been shown to catalyse this step by binding to components of TFIID. Activator binding to its cognate DNA element thus brings TFIID to the promoter. That activators can recruit TFIID to a promoter as compared to DNA bound TFIID recruiting activators to mediate some downstream event is elegantly supported by experiments performed by Klein and Struhl (Klein and Struhl, 1994). A mutant TBP (TBPm3) was expressed in yeast bearing a mutant TATA (TGTA) sequence in front of HIS3 recognised only by TBPm3 and not wild type TBP. The kinetics of HIS3 expression was analysed upon induction of TBPm3 in the presence and absence of GCN4 (HIS3

activator). They showed that HIS3 induction followed the kinetics of TBPm3 induction only in the presence of GCN4. In the absence of GCN4 there was a severe delay in induction of HIS3 upon induction of TBPm3. This suggests that TBP recruitment to a promoter is rate limiting and that activators can serve to enhance this step. Indeed, direct recruitment of TFIID to a promoter without an activator has been demonstrated. Fusion of TBP to a lexA DNA binding domain allows efficient activation of a TATA containing promoter when a lexA operator is present upstream (Chatterjee and Struhl,1995). Thus it appears that the exact mechanism of TFIID recruitment doesn't matter as long as TFIID is drawn to the TATA element. Recruitment of TFIID allows the further recruitment of the RNA holocomplex which contains the bulk of the remaining complexes required to form a competent complex (Koleske and Young,1994; Kim *et al.*,1994). Broadly speaking then, the stability of the fully assembled complex is governed by the binding energies associated with the activator-DNA, activator-TFIID, TFIID-DNA, TFIID-holocomplex and activator-holocomplex interactions. That pre-initiation complex formation occurs via recruitment of TFIID to a DNA bound activator followed by RNA PolII holocomplex recruitment however should be seen in the light of 'Potentiator' mutant experiments (Barberis *et al.*,1995; Farrell *et al.*,1996). GAL11 encodes a protein found associated with the RNA holocomplex. The mutation gal11-P, allows activation of UASG bearing genes by a mutant gal4 which lacks the activation domain. It has been shown that gal11-P interacts with the gal4 dimerisation domain. The implication that this has for the role of activation domains in general is discussed later. The point to note here is that transcription initiation occurs in this case by recruiting the RNA holocomplex rather than TFIID to a promoter. Presumably, activator binding recruits the holocomplex via gal11-P and the regular

holocomplex-TFIID interactions then recruit TFIID to establish a competent initiation complex.

So if a point mutation in GAL11 allows such drastic re-organisation of protein-protein interactions what are the natural targets of activators? Sp1 can be shown to interact with TAF_{II}110 and VP16 interacts with both TFIIB and TFIIH (Ha *et al.*,1993). The array of targets for activators is bewildering with the only real theme being that of multiplicity of interactions exist. Activator by-pass experiments confirm this; tethering a myc tag to gal4 DNA binding domain (DBD) and max to TBP induces activation (Klages and Strubin,1995).

Activators that interact with TFIIB and TFIIH mediate activation via a step beyond recruitment. In the case of TFIIB (a target for acidic activators such as VP16), binding of activator doesn't just cause recruitment but has also to elicit a large conformational change to expose binding sites in TFIIB for PolII and TFIIF (see Klages and Strubin, 1995). In the case of TFIIH, binding must induce helicase and protein kinase activity essential for open complex formation (Xiao *et al.*,1994; Drapkin and Reinberg,1994). If an activator acts to isomerise a prebound core complex from the closed to open state then one can see how a DNA binding domain can be obviated as long as the cellular concentration of the activator is high enough. For example, the bacterial activator NTRC (which lacks a DNA binding domain) acts on a prebound RNA polymerase complex at the *glnAp2* promoter to isomerise to the open state in an ATP dependent manner (Sasse-Dwight and Gralla,1988; Ninfa *et al.*,1987). So in summary, activators can act at the step of core complex-DNA binding or at the step of closed to open state isomerisation. The first has an absolute requirement for a DNA binding domain while the second needs a DNA binding domain only to increase

the local concentration of activator at the required DNA address. Raising the total activator concentration in the cell has no effect on recruitment enhancing activators (and may reduce activation due to titration of co-activating factors) but can aid activation of isomerisation enhancing activators.

Recruitment then, is a major aspect of activator function. Further, as has been stated above, TAFs seem to be required to convey the activator effect to the core complex. However, recent experiments have shown that TAF depletion in yeast though ultimately lethal has little or no effect on activation of TATA containing genes *in vivo* but does abolish activation of TATAless genes (Moqtaderi *et al.*,1996a; Walker *et al.*,1996). How can these two ideas (that of specific activator-TAF interactions and that of TAF independent activation) be reconciled? It may be that there is a large degree of redundancy in the TAF-activator interaction and so removal of one TAF merely allows a different TAF to become a favoured target. Whether the new interaction is as favourable as the original would not be assessable in the experiments performed unless the difference was very marked. Another possibility is that TAF removal allows TBP or other TFII components to become targets. Both possibilities would be consistent with the activator by-pass experiments which show that almost any component of the core complex can become a target for recruitment. TAFs exist to offer favourable interactions with the core complex for certain specific activators. If a gene is regulated by many activator signals then potentially many TAFs are required to mediate their interactions. This could explain the lethality of TAF depletion. Cell cycle regulator genes have many inputs in the form of activators and repressors and thus may require a complement of TAFs to enhance their effects on the core complex. TAF loss would

result in inefficient regulation of these genes and cell death. Indeed, it was found that TAF depletion resulted in cell cycle arrest at very specific points in the cell cycle, consistent with this view. That TAF depletion effects activation of TATAless genes is consistent with their role in enhancing activator interaction and providing greater binding energy to the initiation complex. TAF loss would result in an alternative less avid activator-core complex interaction and thus weaker activation. This effect would be less marked on the tighter bound TATA containing promoter complexes.

Before any member of the initiation complex can approach the promoter it must penetrate the nucleosome structures. Histones have to be remodelled/repositioned to allow access of the transcription machinery to the DNA. Promoter by-pass experiments which do-away with activation domains suggest that activation domains do not possess some inherent ability to re-configure chromatin. Instead it would appear that the core complex and holo-enzyme compete with histones for DNA access. Activators help to shift the binding equilibrium to the complex bound state. In this model then, basal activity arises possibly due to the infrequent 'winning' of the core complex over histones for DNA binding much like basal activity in prokaryotes arises due to the fortuitous successful binding of holo-enzyme and complex opening in the absence of activator.

Activities have been characterised that aid remodelling of chromatin. For example the SWI/SNF complex found in yeast can remodel chromatin and aid activation (Peterson and Tamkun,1995). However, removal of the complex does not alter the maximum activity of PHO5 or GAL1. Removal of 2 UASGs upstream of GAL1TATA causes severe loss of activity in SWI/SNF delete strains (Ptashne and

Gann,1997). This suggests that SWI/SNF remodelling is required to activate promoters that only weakly recruit core complexes. Strong promoters with large binding energies can force the core-complex binding equilibrium adequately to the bound-open state without such help. It would seem then that if a cell is to modulate more than one gene with a single activator then one way of getting different activities is to modulate activator occupancy by having fewer and/or weaker sites. Though this will give the required spectrum of activity, it also makes complex formation more difficult at weaker activation sites in the presence of chromatin. Hence there is a requirement for remodelling activities like SWI/SNF which though dispensable at stronger promoters, is essential at weaker ones. So why have chromatin at all? Promoters are probably too leaky in their unactivated states to be 'naked' and must be packaged to prevent i) the cell from expending unsustainable levels of resources in supporting expression, ii) loss of tight regulatory control due to trace levels of regulatory molecules. So, in summary, chromatin acts to reduce basal activity and is remodelled at activated promoters. This remodelling is aided at the weaker promoters by complexes like SWI/SNF.

The implication here is quite profound in that the mechanism of basal transcription is not fundamentally different from activated transcription - it merely represents the stochastic nature of individual complexes binding to individual DNA addresses. This should be contrasted with the view that because 'auxiliary' components are required to give activation (e.g. TAF_{II}110 is required for Sp1 activation) which are not required for basal transcription, the mechanisms must be different. The extended 'component list' for activated transcription is

probably required to maintain the equilibrium in the bound-open state and probably does not reflect a deep difference in mechanism.

A MOLECULAR ANALYSIS: Repressors

The identification of the lac repressor in *E.coli* by Jacob and Monod in 1961 gave us the first glimpse at how protein synthesis was regulated at the genetic level (Jacob and Monod,1961). In fact the mechanism at play at the lac operon was so elegant that it was tempting to think that this mode of regulation would be a paradigm found at all loci. The role of positive regulation was only really recognised as a bona fide mechanism in the mid-70s in a paper by the group of Englesberg working on the *E.coli* araBAD operon and its activation by araC (Lee *et al.*,1974; reviewed in Englesberg and Wilcox, 1974). The identification of enhancers in 1981 (Banerji *et al.*,1981) and the discovery of Sp1 as mentioned earlier, re-affirmed the notion that in eukaryotes, with their more complex genomes, positive regulation would predominate because it would be more efficient to turn on the genes required rather than turn off those that weren't. Recently, it has become obvious that negative regulation is at least as important in eukaryotes as positive regulation.

Negative regulation can act at any step in the initiation complex formation pathway. Negative regulators can therefore be broadly split into two classes: those that act on activators to prevent activation and those that act on regulatory machinery components other than activators.

Before an activator can activate transcription it must be translocated into the nucleus. Many regulators classed as repressors act at this step to prevent entry of activator to the nucleus. The drosophila

activator Dorsal can be kept out of the nucleus by the protein cactus (Roth *et al.*,1991). Cactus binds the Rel domain of dorsal and interferes with the function of the nuclear localisation signal (Kidd,1992).

Activators are often multisubunit complexes and repressors can disrupt the formation of activator complexes. For example, the activators *daughterless* and *achaete*, *scute*, *asense* genes of the acheate-scute complex (AS-C) encode basic helix-loop-helix (bHLH) proteins which heterodimerise, bind DNA and activate target genes in the sensory organ precursors (Villares and Cabrera,1987; Alonso and Cabrera,1988; Caudy *et al.*,1988; Murre *et al.*,1989). These target genes are silent in epidermal precursors. *Extramachrochaete (emc)* and *hairy (h)* both suppress sensory organ development. Analysis of the structure of *emc* shows it to be a helix loop helix protein which lacks the basic DNA binding domain (Ellis *et al.*,1990; Garrell and Modolell,1990). This suggests a very elegant way of preventing activation. Dimerisation of *emc* with other members of AS-C and *da* thus prevents DNA binding and therefore loss of activation (Van-Doren *et al.*,1991).

Upon activator acquiring the necessary conformation and entering the nucleus it must bind its cognate element in order to specifically activate the target promoter. At this point, the activator may face competition for its binding site with the repressor or with components of silencing machinery set into motion by the action of a repressor signal (see later for discussion on the effects of chromatin and nucleosome structure on gene expression). An example of direct repressor competition can be seen with the action of *Kruppel (Kr)* in its guise as a repressor. *Bicoid (bcd)* and *hunchback (hb)* are two activators that have their elements in the stripe 2 element in the promoter of the drosophila homeodomain protein *eve* (Frasch and Levine,1987;

Stanojevic *et al.*,1989). Virtually all the *bcd* and *hb* sites have an overlapping *Kr* element. It has been shown that *bcd* and *hb* cannot bind their elements if a proximal or overlapping *Kr* element is occupied (Small *et al.*,1991). Thus expression of *Kr* prevents activation of *eve* by *bdc* and *hb*. This is a very local form of repression in that *Kr* binding in the stripe2 element has no effect on activators in the stripe3 element of the same promoter 1.5Kb upstream (Goto *et al.*,1989; Harding *et al.*,1989).

A more elaborate mechanism of activator exclusion through repressor binding is seen in the regulation of the MHCII genes. The repressor YB-1 has its element close to the activator site. YB-1 upon binding DNA induces local unwinding, thereby destroying the activator site (MacDonald *et al.*,1995; Ting *et al.*,1994).

Acquisition of element by the activator is not sufficient to cause gene activation. Contact must be made between the activator's activation domain and the promoter core complex or other components of the activation pathway. Repressors are known that interfere with this step of transcription initiation. The yeast galactose utilisation pathway is potently upregulated by the product of the GAL4 gene, gal4p (Johnston,1987). Amongst other targets, gal4p binds sites in the GAL1/10 divergent promoter and upregulates the promoters over 1000 fold in the presence of galactose. In the absence of galactose, the product of the GAL80 gene binds the gal4 activation domain and prevents gal4p making contact with its target (Johnston *et al.*,1987; Ma and Ptashne,1987a; Ma and Ptashne,1987b; Salmeronet *et al.*,1990). There is evidence that in the presence of galactose, gal80p doesn't actually come away from gal4p but merely undergoes a conformational change to expose the activation domain (Mylin *et al.*,1989; Mylin *et al.*,1990;

Parthun and Jaehning,1992; Chasman and Kornberg,1990; Leuther and Johnston,1992). The protein gal80p does not possess a DNA binding domain but relies purely on protein-protein interactions to exert its effect. However, examples exist of cap activation domains which do require DNA binding sites. For example, the activator c-myc-CF1 upregulates c-myc. The repressor c-myc-PRF shuts down c-myc expression by binding adjacent sites to the c-myc-CF1 elements to mask the activation domain (Kakkis and Calame,1987; Kakkis *et al.*,1989; Riggset *al.*,1991). Repressing factors can also bind adjacent elements to activator sites and modulate activator function in more elaborate ways. For example, the drosophila *zen* promoter contains a number of dorsal binding sites, each with an adjacent ventral repression element. Occupation of these elements by DSP-1 converts *dorsal* from a transcriptional activator to a repressor (Lehming *et al.*,1994).

A more 'active' mode of repression works by modifying the core complex in some way as to prevent transcription regardless of how many activators are bound to upstream elements. This is a very 'global' mode of repression and has the advantage that it can be used on promoters with many activators without the need to have a dedicated repressor for each activator. The repressor *Kr* works via this mechanism as well as through activator exclusion mentioned above. An elegant series of experiments show that *Kr*, at low concentrations activates target promoters by binding the K-element. At high concentration *Kr* homodimerises to become a potent repressor (Sauer and Jackle,1993). It is now established that dimerisation allows *Kr* to interact with TFIIE β , a member of the RNAPII holocomplex (Sauer *et al.*,1995). TFIIE β is involved in the regulation of TFIIH ATPase, helicase and C-Terminal Domain (CTD) kinase activity (Usheva *et al.*,1992; Maxon

et al.,1994). It is likely that one way in which *Kr* affects repression is by preventing hyper-phosphorylation of the RNA PolIII CTD and thus promoter clearance. Such a mechanism would make a core complex immune to all activator signals. Interestingly, the repressor *eve* is also thought to mediate repression via TFIIE β . Although *Kr* mediated repression *in vitro* is inhibited by antibodies to TFIIE β , under the same conditions and same antibody, *eve* can still mediate repression. This shows that two repressors can act on the same component of the core complex but via different mechanisms. Further, *Kr* exemplifies another feature which one sees over and over again in gene regulation: that of multiplicity of function. Not only can *Kr* activate, it can repress. Repression is mediated by i) C-terminal sequences upon dimerisation which allow interaction with TFIIE β ii) exclusion of activator binding and also iii) in mammalian cells, the N-terminal *Kruppel* Associated Box (KRAB) domain is also able to mediate repression, though the exact mechanism is not known.

Whatever the mode of KRAB mediated repression is, it seems to depend on the arrangement of core complex elements. Pengue and Lania showed that a Tet-DBD/KRAB fusion protein could repress a TATA containing promoter activated by a number of different types of activation domain (GAL4, VP16, Sp1, p65, E1a and E2F). However, no significant repression was seen on a Initiator containing TATAless promoter activated by the same activators (Pengue and Lania,1996).

So *Kr* can mediate repression by directly contacting a component of the basal machinery, TFIIE β . It can be said then, that *Kr* has intrinsic repression ability. This should be compared to that class of repressor which recruits other repression machinery to a promoter. The product of the MIG1 gene in yeast is a potent repressor of many genes involved

in carbon source regulation (Nehlin and Ronne,1990). In the presence of glucose, mig1p enters the nucleus and binds the Upstream Regulatory Sequence (URS) in many promoters of the galactose utilisation pathway, sucrose fermentation and gluconeogenesis (Carlson *et al.*,1984; Trumbly,1986; Celenza and Carlson,1984; Celenza and Carlson,1986). mig1p *per se* has no intrinsic repression ability but instead recruits the co-repressor complex SSN6/TUP1 to the target promoter (Keleher *et al.*,1992; Treital and Carlson,1995). One sees a 100% loss of mig1p mediated glucose repression of both the SUC2 and GAL1 promoter in a *ssn6⁻* or *tup1⁻* strain. Further, direct recruitment of *ssn6p* to a promoter by *lexA* fusion results in potent repression in a TUP1 dependent manner. However, direct recruitment of *tup1p* to a promoter results in SSN6 independent repression. This suggests that *ssn6p* and *tup1p* act as a complex that is directed to a target promoter by, amongst others, mig1p and that *tup1p* is probably more directly involved in affecting repression. The nature of SSN6/TUP1 repression is not known but it was recently found that SRB10 and SRB11 were required for full repression by this co-complex (Kuchin *et al.*,1995; Wahi and Johnson,1995). SRB10 is a kinase that acts on the cyclin SRB11. Both have been localised to the RNAPII holo-enzyme (Liao *et al.*,1995). Whether SSN6/TUP1 just use SRB10/SRB11 as 'handles' on the holo-enzyme or whether SSN6/TUP1 can modulate the kinase activity of SRB10 is not clear. It remains to be seen if SSN6/TUP1 can prevent transcription initiation by modulating the kinase activity of SRB10 on SRB11.

Both activation and repression of promoters occurs in the context of packaged DNA in the form of chromatin as compared to naked DNA. As well as regulatory factors acting on the core complex and basal

components, chromatin must be made opaque or transparent to transcription factors according to the transcriptional needs of the cell.

Mutation hunts in yeast have done much to allow the role of chromatin in gene regulation to be dissected. Screens to isolate regulators of SUC2 identified a number of mutations which abolished SUC2 expression under derepressing conditions (Neugeborn and Carlson,1984). Two mutations, SNF2 and SNF5 (SNF=Sucrose Non Fermenter) were found to be identical to SWI2 (Laurent *et al.*,1991), (Yoshimoto and Yamashita,1991) and SWI10 (Winston and Carlson,1992) (SWI=SWItch) respectively, genes important in the regulation of HO recombinase - a gene involved in mating type switch (Nasmyth,1983). A supressor of SNF2, SNF5 and SNF6 (another SUC2 non-fermenting mutant), SSN20 (Suppressor of Sucrose Non-fermenter) (Estruch and Carlson,1990; Neugeborn *et al.*,1986) was found to be identical to SPT6 (SuPpressor of Transposition) a gene functionally related to HTA1 and HTB1. HTA1 and HTB1 encode Histone 2A and Histone 2B respectively (Clark-Adams *et al.*,1988). These analyses showed that histones were involved in the regulation of a diverse group of genes in yeast ranging from carbon utilisation to mating-type switching. SWI2/SNF2, SNF5 and SNF6 do not possess any DNA binding domain and thus, as activators are unlike those discussed earlier. A *lexA* fusion of SWI2/SNF2 gave activation of heterologous promoters in a SNF5, SNF6 and SWI1 dependent manner (Laurent *et al.*,1991). *lexA*-SNF5 yielded similar results requiring the other mentioned factors. However, *LexA*-SNF6 gives activation independent of SWI2/SNF2, SNF5 or SWI1. This suggests that these gene products act as a complex with SNF6 being more directly in contact with the activation apparatus (Winston and Carlson,1992). As mentioned earlier, though GAL1

activation by gal4p doesn't seem to be affected by mutation of SWI2/SNF2, activation from a minimal UASG containing GAL1 promoter is severely diminished. Further, activation of yeast promoters by both drosophila activators *fushitirazu* (Peterson and Herskowitz,1992) and *bicoid* (Winston and Carlson,1992) are SWI dependent. Also, lexA fusions of the gal4p, *ftz* and *bc* depend on SWI in a manner depending on number of elements, target promoter and actual activator. Arguably, the most interesting point however is that activation by glucocorticoid receptor in yeast is SWI/SNF dependent (see Peterson, 1992). This point is discussed later with respect to the requirement of co-repressors in mediating repression by unliganded, nuclear receptors.

The above examples are of mutations in histone related genes that abolish activation of other genes. There are other mutations in a different class of histones that relieve the repressing effects of SWI and SNF mutations. SIN1 and SIN2 mutations suppress *swi2/snf2*. SIN1 is a member of the High Mobility Group (HMG) family (Kruger and Herskowitz,1991) while SIN2 encodes Histone H3 (Kruger and Herskowitz, unpublished data). The mutation *sin3(=rpd1)* suppresses *swi5* and also *ime1* (IME1 is an activator of IME2, a gene involved in the induction of meiosis). What this means is that although *ime1*⁻ cells cannot undergo meiosis, *sin3* mutants can undergo meiosis in an *ime1*⁻ background. SIN3-lexA fusions can repress activated heterologous promoters (Wang and Stillman,1993) The protein has 4 Paired Amphipathic Helices (PAHs), motifs implicated in protein-protein interactions (Wang *et al.*,1990). PAH3 is absolutely required for sporulation and repression of the HO promoter while the remaining 3 may be partially redundant (at least for those effects assayed). The PAHs are similar to the Helix-Loop-Helix (HLH) and Trico-Peptide-

Repeat (TPR) motifs found in other transcription factors such as MyoD, myc and SSN6 respectively (Goebel and Yanagida,1991; Hirano *et al.*,1990; Murre *et al.*,1989; Sikorski *et al.*,1990; Voronova and Baltimore,1990). While MyoD has one HLH motif, SSN6 has 10 TPRs. In yeast, Sin3p binds the DNA binding transcription factor encoded by the UME6 gene (Kadosh and Struhl,1997). It would seem then that these DNA binding repressors recruit Sin3p to promoters to seed 'local repressing chromatin changes'. The exact nature of this 'local repressing chromatin change' has come to light in some excellent experiments reported recently.

In mammalian systems, the proteins Mad and Mxi1 bind Max to prevent the interaction of Max and Myc to form the max-myc activator. As well as disrupting activator formation, both Mad and Mxi1 possess a Sin3 Interaction Domain (SID) (Henriksson and Luscher,1996). In mice there are two known isoforms of the yeast orthologue SIN3, mSin3A and mSin3B (Ayer *et al.*,1996; Ayer *et al.*,1995; Schreiber-Agus *et al.*,1995). Both have been shown to bind the Mad/Mxi SID. In 2-hybrid screens, mSin3B was found to interact with N-CoR (see later) and also, immunoprecipitation experiments pulled down mammalian RPD3 (Alland *et al.*,1997). RPD3 was isolated along with SIN3(=RPD1) as an extragenic suppresser of swi5 and encodes Histone de-acetylase (HD1) (Nasmyth *et al.*,1987; Vidal and Gaber,1991; Stillman *et al.*,1994). Further, RPD3 or SIN3 loss in yeast results in derepression of the same set of genes suggesting that they function in the same regulatory pathway. This is consistent with RPD3 and SIN3 being found in a complex. Also, consistent with yeast data, PAH3 and one other PAH are essential for repression of basal activity of heterologous promoters in transient transfection assays in HeLa cells (See discussion on targets of

acetylation/de-acetylation activity and relevance to transient transfection assays in 'Discussion').

N-CoR was originally isolated as a co-repressor required for the repression ability of un-liganded thyroid receptor and now has been shown to be required for Mad/Mx1 mediated repression (Heinzel *et al.*,1997). However, micro-injection antibody analysis shows that the domains required for these two functions are different, with the C-terminus being required for Mad/Mx1 repression and the N-terminus for nuclear receptor repression.

The SIN3/RPD3/N-CoR complex has potent histone de-acetylase activity. Histone de-acetylation is known to be involved in formation of transcriptionally quiescent chromatin. The picture that has come to light then, is that Mad/Mx1 and nuclear receptors mediate repression by recruiting histone de-acetylation activities to the target promoter. This mode of repression is obviously conserved through yeast to mammals.

The picture becomes more interesting in the case of the nuclear receptors. Upon ligand binding, it has been shown that the dimerised receptors recruit the co-activator N-CoA1/SRC-1 (steroid receptor co-activator) and GRIP-1/TIF-1 (Torchia *et al.*,1997; Heery *et al.*,1997). SRC-1 is associated with the general activator CREB Binding Protein (CBP). CBP acts as a general integrator of many cellular signals such as those from the JAK/STAT pathway (Bhattacharya *et al.*,1996; Zhang *et al.*,1996), JUN and CREB (Kwok *et al.*,1994; Arias *et al.*,1994). CBP itself is associated with histone acetyl transferase (HAT) activity (Bannister and Kouzarides,1996). Histone acetylation is known to induce active chromatin configurations by opening up tightly packed nucleosomes. In

summary then, nuclear receptors in the unliganded state recruit histone de-acetylase activity to target promoters to induce silent chromatin states. Upon ligand binding, histone de-acetylase activity is exchanged for histone acetylase activity to induce local unwinding of chromatin and thus access by the general transcription machinery to the target promoter. In this case both repression and activation are active events mediated by the same molecule to provide very tight regulation.

The sum of all of the above mentioned mechanisms plus all the multitude of as yet uncharacterised processes act in concert throughout the genome to give rise to the phenotypic diversity seen within cells, whole organisms and the nervous system in particular. One theme becoming apparent in the establishment of the neuronal phenotype is the use of the RE1/NRSE (Restrictor Element/Neural Restrictive Silencing Element) element found in a number of neural specific gene promoters to restrict expression of these genes to the nervous system.

The RE1/NRSE element was originally identified in the type II Na⁺ channel and SCG10 promoters and was shown to be responsible for silencing gene expression in non-neural cells. Since then DNA data base analysis has identified this element in 18 genes, most of which are selectively expressed in neural tissue. The sequence shows very high homology in various genes and is well conserved in species as diverse as human and chicken. However, functional characterisation of this element has only been demonstrated for promoters of the sodium type II channel gene (Maue *et al.*, 1990), the SCG 10 gene (Mori *et al.*, 1990), the Dopamine β -Hydroxylase gene (Ishiguro *et al.*, 1993) the Na, K-ATPase α 3 subunit (Pathak *et al.*, 1994) and the synapsin I gene (Li *et al.*, 1993; Howland *et al.*, 1991; Schoch *et al.*, 1996) In the case of the human synapsin I gene, Schoch *et al.* dissected the promoter and

concluded that the RE1/NRSE binding motif was solely responsible for the neural specific expression of this gene (Schoch *et al.*,1996). In addition, a transgenic analysis of the RE1/NRSE site has been reported for two genes, the Na,K-ATPase α 3 subunit (Pathak *et al.*,1994) and SCG10 gene (Wuenshell *et al.*, 1990). In the case of the Na,K-ATPase α 3 subunit, 210bp of the sequence 5' to the transcription initiation site drove tissue-specific expression. Mutation of the RE1/NRSE site within this region, resulted in ectopic expression of the reporter gene in all non-neuronal tissues tested. Subsequently however, work carried out in the Anderson lab has shown that the RE1 sequence from the Na,K-ATPase α 3 subunit gene is not able to bind recombinant REST/NRSF (see below) in gel shift assays (Schoenherr *et al.*, 1996). It is therefore likely that this gene does not contain a true REST/NRSF binding site and that the putative RE1 element binds another activity in cells. In the case of the SCG10 gene, deletion of the 5'-most 3.7Kb (and subsequent removal of the RE1/NRSE) yielded ectopic expression of the transgene in numerous nonneuronal tissues. However expression did remain highest in the brain. A transgene containing 4Kb of 5' sequence (encompassing the RE1/NRSE) was expressed specifically in neuronal cells with no ectopic expression in non-neuronal cells.

The RE1 element was subsequently found to bind the zinc finger repressor REST/NRSF (Chong *et al.*, 1995; Schoenherr and Anderson,1995). Primary structure analysis of REST shows it to encode a 116Kd protein with 8 clustered Kruppel like C2H2 class Zinc fingers (residues 160-412) each containing a non-canonical tryptophan residue. A single zinc finger is found just upstream of the C-terminus. A region stretching from residue 440 to 600 is 25% lysine and a proline rich region extends from residue 600-800. Recently, it was shown that 2

distinct repression domains existed within REST: The N-terminal 73 amino-acids and the C-terminal Zinc finger (Tapia-Ramirez *et al.*,1997). Neither domain showed homology to previously characterised repression motifs e.g. a KRAB domain or SID (see earlier). Interestingly, in transient transfection assays, the proline rich motif showed no repression ability and the lysine rich region showed only marginal repression properties. These points are discussed further in 'Discussion'. How REST mediated repression upon acquiring the RE1 element is not clear; protein targets of REST have previously not been described.

YEAST: A Model System.

A recurring theme in the above discussion is the very large degree of structural and functional conservation between yeast, flies, worms and mammals of the general transcriptional apparatus. For example, the TFIID complex shares homology throughout the phyla (Moqtaderi *et al.*,1996b) and *in vitro* at least many components can be exchanged between organisms and yet maintain function. Most TAFs are found throughout the phyla with the exception of TAF_{II}110 which is not found in yeast (and may incidentally explain the inability of glutamine rich activators to function in yeast).

One tends to see functional themes present in yeast that are also present in higher eukaryotes, only expanded. For example, yeast posses the gene ySIN3. Yeast SIN3 has a mouse homologue: mSIN3A and mSIN3B. In mice, the activity associated with SIN3 has been partitioned into 2 isoforms of the same protein thus an increase in diversity in higher eukaryotes is seen compared to that in yeast.

It is this conservation of genes and mechanisms between yeast and the higher eukaryotes coupled with the immense ease of genetic

manipulation that has been exploited to unravel the transcriptional mechanisms and molecules that guide the establishment of cellular phenotype.

Muscarinic Receptor Gene Regulation

The degree of cellular diversity seen in the mammalian nervous system is unmatched in any other organ and G-protein coupled receptors probably represent the largest component of this diversity representing as much as 1% of all genes. As, this gene family represents a model system for identifying molecules and mechanisms involved in the establishment of differentiated neuronal phenotype.

GPRs are responsible for a large portion of signal transduction in the nervous system and are encoded by one of the most diverse gene families in the mammalian genome. Most, if not all, of these gene products have a unique distribution within (and without) the nervous system. An important question, therefore, is what determines the receptor repertoire of individual neurones? Of the several hundred members of the G-protein coupled receptors very little is known about the gene structure and the transcriptional control regions that result in this tightly controlled expression (see Wood and Buckley (1995) *Forme* 5, 29-38). In order to redress this lack of information five muscarinic receptors (m1-m5) were identified and cloned (Kubo *et al.*,1987; Bonner *et al.*,1987; Bonner *et al.*,1988). Each has a unique pharmacological profile and pattern of expression within the CNS (Peralta *et al.*,1987; Buckley *et al.*,1988; Buckley *et al.*,1989). The m4 gene is expressed mainly in telencephalic regions of the central nervous system (Buckley *et al.*,1988), and autonomic ganglia (Hassal *et al.*,1993; Brown *et al.*,1995) and has also been found in rabbit lung (Mak *et al.*,1993;

Lazareno *et al.*,1990). Effects mediated by activation of m4 receptors include; closing of N-type voltage sensitive Ca^{2+} channels, activation of K^{+} channels and inhibition of adenylyl cyclase. In order to gain insight into the mechanisms that determine the expression profile of GPRs the lab recently isolated a cosmid clone containing the gene for the m4 cholinergic muscarinic receptor and characterised its gene structure (Wood *et al.*,1995). The gene encoding the rat m4 receptor consists of two exons, an upstream non-coding exon which is separated from a coding exon by a 4.8kb intron. In this study I show that a genomic fragment containing 1440bp of 5' sequence and 80-270 bp of the first exon is capable of driving cell specific expression *in vitro* . I have identified DNA elements responsible for regulating cell specific expression of the m4 gene. In collaboration with others in the group, gel mobility shift assays have been employed to identify genomic elements in the m4 promoter that are capable of recruiting DNA binding proteins.

After characterisation of the *cis*-elements in the m4 promoter and the elucidation of the role that the neural specific repressor REST/NRSF plays in regulating m4 expression, I describe the use of novel yeast based strategies used to illuminate the molecular mechanisms that underlie REST mediated repression. In doing so I implicate the evolutionary conserved co-repressor gene SIN3 in the repression pathway REST uses to affect silencing on its target genes and the consequences this has for our understanding of the role of acetylation/de-acetylation in gene regulation.

Finally I discuss future directions that I intend to pursue to carry these studies further and more fully describe those mechanisms that lead to the establishment of cellular phenotype in general and neuronal phenotype in particular.

CHAPTER 2

MATERIALS AND METHODS

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CLONING PROCEDURES.

Cloning of m4 Promoter Deletion Constructs used in Chapter 3.

All m4 promoter fragments proved refractory to cloning into the Luciferase reporter plasmid pGL3 giving rise to deleted and re-arranged plasmids in JS5 {*araD139, Δ(ara, leu)7697, Δ(lac)χ74, galU, galK, hsdR2(rk'mk'), mcrA, mcrBC, rpsL(Str^r), thi, recA1/F':::Tn10(Tet^r)proAB, lacI^q, LacZDM15*} (Bio-Rad) , XL1-Blue {*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac/F':::Tn10(Tet^r)proAB, lacI^q, LacZDM15*} (Stratagene) and SURE {*e14-(McrA-)Δ(mcrCB-, hsdSMR-, mrr)171endA1, supE44, thi'1, gyrA96, relA1, lac, recB, recJ, sbcC, umuC::Tn5(Kan^r)uvrC/Tn10(Tet^r)proAB, lacI^q, LacZDM15*} (Stratagene) cells. Therefore I removed the high copy pUC origin from pGL3 and replaced it with the low copy p15A origin from pACYC177.

The low-copy origin for pGL3LC (pGL3 Low Copy) was generated by PCR using pACYC177 (New England Biolabs), as template and the oligonucleotides pACYC177-1597a (5'-GTCTGGATCCgtcgacCACCT-TCTTACGAGGCAGACC-3') and pACYC177-4s (5'-ACGCCGGGCAAGAGCAACTCGGTCG-3'). The oligonucleotide pACYC177-1597a has a BamH1(underlined)-Sal1(lowercase) linker. PCR was performed using Taq and Taq-extender (Stratagene) on a Hybaid Thermal cycler. The plasmid pGL3LC was made by cloning the BamH1/Bbu1 p15A origin fragment of the pACYC177-4s/pACYC177-1597a PCR product into BamH1/Bbu1 cut pGL3basic(Promega).

Preparation of pGL3LC plasmid DNA (and constructs cloned therein) was performed using a modified version of the Qiagen tip-20 mini-prep protocol. Briefly, 50 mls of overnight LB-Amp (50µg/ml) culture inoculated with a single colony was pelleted and resuspended in 4mls Resuspension Buffer P1 (100ug/ml RNase A, 50mM Tris/HCl, 10mM EDTA, pH8.0). After addition of 4mls Alkaline Lysis Buffer P2 (200mM NaOH, 1% SDS) and incubation for 5 minutes at room temperature, 4 mls of Neutralisation Buffer P3 (3.0 M KAc, pH5.5) was added and incubated for 10 minutes on ice with occasional inversion. The solution was then spun at 6700g and the supernatant poured down a Qiagen Tip-20 column pre-equilibrated with Equilibration Buffer (750mM NaCl, 50mM MOPS, pH 7.0, 15%(v/v) isopropanol, 0.15%(v/v) Triton X-100). The column was then washed with 2mls Wash Buffer (1.0M NaCl, 50mM MOPS, pH 7.0, 15%(v/v) isopropanol) and eluted in 0.8mls Elution Buffer (1.25M NaCl, 50mM Tris-Cl (pH 8.5), 15%(v/v) isopropanol). The eluant was precipitated with 0.7 volumes isopropanol by centrifugation at 6700g for 30 minutes at 4°C. The pellet was washed with 1ml 70% (v/v) ethanol by centrifugation at 6700g for 10 minutes at room temperature. The supernatant was removed and the pellet allowed to air dry for 10 minutes. The pellet was resuspended in 50µl TE (10mM Tris-Cl (pH 8.0), 1mM EDTA).

REPORTER PLASMID CONSTRUCTION.

Primers to sequences of the m4 promoter {Rm4/-1440s(5'-GAAG-GTACCGGATCCCTTACACATTACAACC-3'), Rm4/-677s (5'-GAAGGTACCGT-GACCAGTACTCAGGGCCGGG-3'), Rm4/271s(5'-GAAGGTACCCTCCAGTCTTT-TGGCTGCCC-3'), Rm4/-151s (5'-GAAGGTACCGGATTAAACTGCAGGCGGG-3'), and Rm4/-50s (5'AGGTACCCTCATAGCCAAGCTTGGCCGCCTG-3')}, containing Acc65I linkers (underlined) were used in conjunction with

Rm4/+80a(5'-GAAGATCTGCCACAGTCGCCAGGGAGGATTAG-3') containing a BglIII linker (underlined) in PCR to amplify fragments of the m4 promoter ranging in size from 130bp to 1520bp. PCR was performed on a Hybaid Thermal Cycler in the presence of Taq (0.4U/ μ l final) and Taq Extender (Pfu polymerase (0.4U/ μ l final)) (Stratagene) to minimise mutations. These were cloned into Acc65I/Bgl II cut pGL3LC to generate the pGL3LC-1440/+80 to pGL3LC-50/+80 series of constructs. In order to construct the pGL3LC -151/+80RE1s and pGL3LC-151/+80RE1a two complimentary oligonucleotides were synthesised to the region between -574 and -550 of the m4 promoter {Rm4RE1s(5'-GTACGGAGCTGTCCGAGGTGCTGAATCTGCCT-3') and Rm4RE1a(5'-GTACAGGCAGATTCAGCACCTCGGACAGCTCC-3')} and constructed such that when annealed they contain Acc65I cohesive ends. These oligonucleotides were cloned into the Acc65I site of pGL3LC-150/+80 and recombinants screened for orientation.

The plasmid -677/+208 was generated by using PCR primers Rm4/-151s and Rm4/+438a (5'-TCTTTCTGCTGGTGCCGGACAGAC-3') to amplify a 589bp fragment from a cosmid containing rat m4 sequence. This was digested with PstI and the 453bp fragment (from -143 to +19) cloned into PstI cut pBluescript KSII⁺ (Stratagene). The insert was sequenced to assess orientation and integrity of sequence. A clone containing a PstI fragment oriented in the T3.-143/+19.T7 direction was digested with HindIII and the resultant 373bp liberated band was cloned into HindII cut pGL3LC-677/+80. The resultant plasmid pGL3LC -677/+208 was sequenced to assess orientation and integrity of clones. The plasmid pGL3LC-151/+208 was generated in the identical manner to pGL3LC-677/+208 but with the HindIII fragment cloned into HindIII cut pGL3LC-151/+80. pGL3LC-677/+208 Δ TSS was generated by using

PCR primers Rm4/+20s (5'ATATATAAAGCTTGGACAGCCCCACCCCACCCC-CGGAGG-3') and Rm4/+208a (5'-ATATATAAAGCTTCTGCAGCTCCGGGCGCTGTGTGG-3') to amplify sequence from rat m4 containing cosmid DNA. The resultant PCR product was digested with HindIII (HindIII linker sequences underlined) and cloned into HindIII cut pGL3LC-677/+80. pGL3LC-151/+208 Δ TSS was made in an identical manner except that the HindIII digested PCR product was cloned into HindIII cut pGL3LC-151/+80. Both pGL3LC-677/+208 Δ TSS and pGL3LC-151/+208 Δ TSS were sequenced to verify integrity of clones. Plasmids pGL3LC-677/+208 Δ TSS and pGL3LC-151/+208 Δ TSS thus contain a deletion spanning 33bp upstream of the putative upstream transcription start point and 22bp downstream.

All constructs generated by PCR were sequenced on an ABI 377 Automated sequencer using an ABI Prism sequencing kit and Hybaid thermal cycler upon cloning into pGL3LC with primers RV3 (5'-CTAGCAAATAGGCTGTCCC-3') and GL2 (5'-CTTTATGTTTTGGCGTCTTCCA-3') (Promega).

Cloning of Constructs Used in Chapter 4.

The reporter plasmid for 1-hybrid library screening, pBM2389.(RE1)₃ was generated by cloning a double stranded oligonucleotide into the EcoR1 site of pBM2389 (Liu *et al.*, 1993) containing RE1 sequences derived from the m4 promoter. The sequence of the oligonucleotides are m4RE1s (5'-AATTCGAGCTGTCCGAGGTGCTGAATCTGGGAGCTGTCCGAGGTGCTGAATCTGCCTG-3') and m4RE1a(5'AATTCAGGCAGATTCAGCACCTCGGACAGCTCCCAGATTCAGCACC

TCGGACAGCTCG-3'). Orientation and integrity of the clone was verified by sequencing with primer GAL1s.

For 1-Hybrid analysis of REST for m4RE1 and NaIIRE1 sequences a double stranded oligonucleotide containing the m4RE1 {5'-TCGAGGAGCTGTCCGAGGTGCTGAATCTGCCT-3' and 5'-TCGAAGGCAGATTCAGCACCTCGGACAGCTCC-3'} sequence and NaII sequence {5'-TCGAGGTGCTGTCCGTGGTTCTGAA-3' and 5'-TCGATTCAGAACCACGGACAGCACC-3'} were cloned into the Xho1 site in the reporter plasmid pBM1933 (CYC1-LACZ{2m/URA3}). The high copy effector plasmid pACT2.DomNeg (REST DNA Binding domain-GAL4 activation domain{2m/LEU2}) was generated by cloning an EcoR1 fragment containing the DNA binding domain (residues 143-446) from pMT.DomNeg (all pMT derived plasmids are gifts from Dr David Anderson, Caltech - Pasadena) into the EcoR1 site of pACT2 (Clontech). The plasmid expressing the REST DNA binding domain to the gal4 activation domain at low levels in yeast, pRS423/REST21-1, was generated by cloning a 2Kb HindIII fragment from 1-hybrid candidate HM-21-1 into the HindIII site of pRS423(Sikorski and Heiter,1989). (A HindIII fragment encompassing the coding region of the yeast URA3 gene when cloned into this site results in low levels of ura3p expression. Cloning of coding fragments in this site should therefore allow expression at low levels.)

Cloning of Constructs Used in Chapter 5.

The expression plasmid pLeonov(HX){Leu2/2m} was generated by removing a HindIII fragment encompassing the GAL4 activation domain and polylinker from pGAD10 (Clontech) and replacing with the double stranded oligonucleotide pLeonov.MCS. pLeonov.MCS was made

by annealing the oligonucleotides pLeonov.MCSs (5'-AGCTTGAATTCGCGGCCGCGGATCCCTCGAGT-3') and pLeonov.MCSa (5'-AGCTACTCGAGGGATCCGCGGCCGCGAATTCA-3').

pRS316.ADHp/t{URA3/Cen} was generated by replacing the PvuII fragment of pRS316 with the SphI fragment encompassing the ADH promoter/terminator cassette of pLeonov(HX). pLeonov.HZ4 was generated by cloning a HindIII/XhoI from pMT.HZ4 containing a fusion of 6 myc tags on the N-terminus of HZ4 into pLeonov. pLeonov.DomNeg was generated by cloning an EcoRI fragment containing the DNA binding domain of mREST (residues 143-446) from pMT.DomNeg into the EcoRI site of pLeonov. pRS316.ADHp/t.HZ4 and pRS316.ADHp/t.DomNeg were generated by gap rescue of XhoI cut pRS316.ADHp/t by pLeonov.HZ4 and pLeonov.DomNeg in the yeast strain FM205. Gap rescue was performed by transforming 2×10^8 yeast cells with 100ng XhoI cut pRS316.ADHp/t and 1 μ g SphI digested pLeonov.HZ4 or pLeonov.DomNeg. Yeast colonies were assayed for recombinants by shuttling plasmids into JS5 Ecoli cells by electroporation of yeast lysates generated by freeze/thaw and subsequent restriction analysis.

pLeonov.HZ4D1-138 was generated by digesting pLeonov.HZ4 with NcoI and BglII, filling in with T4 polymerase and religating. This removes one of six myc tags along with the first 138 residues of HZ4 but maintains the open reading frame. The GAL1-HIS3 reporter plasmid pBM2389(RE1)₃cUAS{TRP1/Cen} was generated by cloning a double stranded oligonucleotide containing a single consensus GAL4 binding site into the unique BamHI site in pBM2389(RE1)₃. The GAL4 consensus site double stranded oligonucleotide was generated by annealing cUASs (5'-GATCGCGGACTGTCCTCCGG-3') and cUASa (5'-

GATCCCGGAGGACAGTCCTCCGC-3'). pRS306.SIN3 was generated by cloning a Xho1/BglIII cut 1783bp PCR fragment of yeast SIN3 (Xho1 site provided by primer SIN3.2861s and BglIII site found in yeast SIN3 coding sequence) into Xho1/BglIII cut pRS306{URA3 integrative}. PCR was performed with primers SIN3.2861s (5'-AGAGACTCGAGGAACGTCTCTCACTGAACAG-3' Xho1 linker underlined) and SIN3.4644a (5'- CATTTCGGAAAGTTGACTCG-3').

CELL CULTURE, TRANSFECTIONS AND PROMOTER ASSAYS.

NG108-15 and CHO cells were cultured in 5% CO₂ at 37°C in DMEM (Gibco) containing 6g/l penicillin, 10g/l streptomycin and 2mM L-glutamine, 0.1mM proline, supplemented with either 10% foetal calf serum (FCS). Neuro2a and 3T3 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 5g/l penicillin, 10g/l streptomycin and 2mM L-glutamine. PC12 cells were maintained in α -MEM medium supplemented with 5% fetal calf serum, 5% horse serum, 5g/l penicillin, 10g/l streptomycin and 2mM L-glutamine. NBOK1 cells were maintained in RPM-1 medium containing 10% fetal calf serum, 2mM L-glutamine, 5g/l penicillin and 10g/l streptomycin.

All transfections for promoter deletion analysis were performed in 24X15mm well trays using Qiagen column purified DNA and lipofectamine (Life Technologies Inc) as described previously (Wood *et al.*,1995). Briefly, 125ng of each m4 plasmid and 125ng of the β -galactosidase expressing CMV-GAL plasmid were incubated with 1.25 μ l lipofectamine in 50 μ l optimem for 30 minutes at room temperature. A further 150 μ l optimem was added and the whole 200 μ l added to a well of cells prewashed with PBS. After 24 hours at 37°C the cells were

washed and fed with 1ml of medium. Cells were harvested 24-48 hours post feeding.

3T3 cells for REST quenching co-transfection experiments were transfected at around 10-20% confluence with 125ng reporter plasmid, 1ng Renilla expressing CMV-Renilla (Promega) plasmid and 125ng gel purified/electro-eluted and precipitated PCR product. RE1 site containing PCR product was generated by amplification of the plasmid pBM2389(RE1)3cUAS with primers GAL1s and GAL1.789a to yield a 500bp fragment containing a tandem array of 3 RE1 sites where each site represents sequence corresponding to -574 to -550 of the rat m4 promoter sequence. The remaining sequence of the PCR product corresponds to flanking vector sequence. 'Blank' PCR product lacking RE1 sites was generated as above using pBM2389 as template. For all transfection experiments cells were harvested 24-48 hrs post-transfection into Reporter Lysis Buffer (Promega) and freeze thawed once. Luciferase activity was assayed using the Promega Luciferase Assay System in a Turner TD-20e luminometer as per manufacturer's instructions.

WESTERN BLOT ANALYSIS.

Protein was extracted from 10mls of overnight yeast culture grown in SD-TRP-LEU to OD₆₀₀=2-5. The cells were harvested and transferred into a 1.5ml eppendorf tube. To the cell pellet 300mg of acid washed glass beads (0.5-1mm diameter) (SIGMA) and 200 μ l Protein Extraction Buffer (100mM KCl, 5mM MgCl₂, 20mM HEPES-pH7.6, 8% glycerol) were added and the samples vortexed vigorously for 1 minute at 4°C. 10 μ g protein was subjected to SDS-PAGE in a 10% gel and transferred to nitrocellulose using a Biorad protein mini-gel

apparatus. The filter was blocked for 2 hours with 5% fetal calf serum, 0.1% Tween-20 and 10% low fat Marvel powdered milk in Phosphate Buffered Saline (PBS). The filter was washed thrice for five minutes each in PBS with 0.1% Tween-20 and then incubated with a 1:1000 dilution of anti-gal4 Activation Domain antibody (Clontech) or anti-myc (9E10 fraction) antibody in PBS with 0.1% Tween-20 for one hour. The filter was washed as described above and incubated with a 1:1000 dilution anti-mouse IgG antibody conjugated to Horse Radish Peroxidase (Amersham Life Sciences) for 1 hour. The filter was washed again three times and exposed to ECL reagent for 1 minute as per manufacturers instructions (Amersham Life Sciences). The filter was exposed to film for 1 minute.

β-GALACTOSIDASE ASSAYS

β-galactosidase assays were performed on disrupted yeast cells and cell lines (harvested in Reporter Lysis Buffer as described above) as follows: 10 mls overnight yeast culture at OD₆₀₀=0.6 were harvested and washed in 25mls water. The pellet was resuspended in 0.5mls PBS and freeze thawed 10 times between liquid nitrogen and water at 37°C. 50μl of lysate was assayed with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as described in Sambrook et al (1989). Values were normalised to cell density and time of reaction

YEAST TRANSFORMATIONS.

All transformations were performed using the method of Schiestl and Geitz (Schiestl and Gietz,1989). Briefly, yeast were grown in the appropriate selection media overnight in 50 mls to a density of 2X10⁹ cells/ml. Cells were harvested and resuspended in 100mM Lithium Acetate (LiOAc) to a final volume of 500μl. 50μl of cell suspension were

transferred to a fresh tube and pelleted. The supernatant was removed and replaced with 240 μ l 50% (w/v) Poly Ethylene Glycol (PEG-3300), 36 μ l 1M LiOAc, 25 μ l boiled salmon sperm DNA (2mg/ml) and 100-500ng plasmid DNA in 50 μ l total volume. The cells were vortexed for 1 minute and incubated at 30°C for 30 minutes and then transferred to 42°C for 30 minutes. The cells were pelleted briefly and resuspended in 100 μ l water and plated onto the appropriate selection media plates.

1-HYBRID LIBRARY SCREENING.

One hundred to 250 μ g of each library was transformed into the yeast strain SFY526{pBM2389(RE1)₃} (SFY526 genotype: Mat a, ura3-52, his3 Δ 200, ade2-101, lys2-801, trp1-901, leu2-3/112, can^r, gal4 Δ 542, gal80 Δ 538, URA3::GAL1-lacZ) and plated out onto selective media using the above transformation procedure scaled up 10 fold. Colonies arising on histidine drop-out plates were picked into 3mls SD-LEU broth and grown overnight. Cells were harvested and lysed with glass beads as described above into 500 μ l DNA Extraction Buffer (10mM Tris-pH 8.0, 1mM EDTA, 100mM NaCl) and 500 μ l Tris Buffered phenol. The samples were centrifuged at 6700g for 5 minutes and the supernatant ethanol precipitated. The washed pellet was resuspended in 100 μ l TE and 1 μ l used to transform JS5 E.coli. Resultant plasmids were sequenced on an ABI 377 automated sequencer with primers 5'AD {5'-CTATTCGATGATGAAGATACCCACCAAACCC-3'} and 3'AD {5'-GTGAACTTGCGGGGTTTTTCAGTATCTACGAT-3'}(Clontech). The library plasmid was re-transformed into the above strain to test for library dependence.

YEAST STRAIN CONSTRUCTION

The yeast strain FM242 (Mat α , *ura3-52*, *lys2-801*, *ade2-101*, *his3 Δ 200*, *trp1 Δ 63*, *leu2 Δ 1*, *cyh2*, *kar1 Δ 15*) and FM205 (Mat α *ura3-52* *his3 Δ 200* *ade2-101* *lys2-801* *trp1 Δ* LEU2⁺ SSN6 Δ 9) were gifts from Mark Johnston, Washington University, St Louis. FM242.*sin3::URA3* was generated by linearising pRS306.SIN3 at a unique Cla1 site within the SIN3 sequence, transforming into FM242 and plating on plates lacking uracil. Colonies were subject to PCR with primers T7 and SIN3.2824s (5'-GGGATAGCGAATGAAAATCC-3') to confirm SIN3 disruption. The resultant strain FM242.*sin3::URA3*, was transformed with pBM2389(RE1)₃cUAS and either *pleonov.HZ4*, *pLeonov.DomNeg* or empty *pLeonov(HX)* and maintained on plates lacking tryptophan, leucine and uracil.

REPRESSION ASSAYS.

Transformants were assayed for REST repression by plating onto plates lacking tryptophan, leucine, uracil and histidine supplemented with various concentrations of 3-AT. Carbon source was either 2% galactose+2% raffinose or 2% glucose. The plates were incubated at 30°C for 3 days and then growth assessed.

CHAPTER 3.

TRANSIENT TRANSFECTION ANALYSIS OF m4 PROMOTER DELETION CONSTRUCTS

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INTRODUCTION

The gene encoding the rat m4 receptor consists of 2 exons, an upstream non-coding exon which is separated from a coding exon by a 4.8kb intron (Wood *et al*,1995). RNase protection assays and primer extensions were initially attempted to ascertain the transcription start site(s) but proved very difficult to interpret due to regions of GC rich sequence and putative secondary structure within the 5' region of the m4 gene. As a result, nuclear run-on assays and northern blots with fragments of the R3-6 cosmid (which encodes the m4 gene) were used to narrow down the junction between promoter sequence and the first exon. The transcription start site was then further mapped via an RT-PCR based approach by reverse transcribing RNA from m4 expressing cell lines (PC12 and NG108-15) and performing PCR with primers pairs around the 5' region of the first exon. Using the nomenclature in Wood *et al*,1995, primers Rm4X4s and Rm4X6a generated a PCR band from reverse transcribed PC12 cDNA but Rm4X10s and Rm4X6a did not. As a result it was concluded that the transcription start site was situated in a 48bp region between primers Rm4X10 and Rm4X4 (see figure 1 of Wood *et al*, 1995)

In this study I show that a genomic fragment containing 1440bp of 5' sequence and 80-270 bp of the first exon is capable of driving cell specific expression *in vitro*. I have identified DNA elements responsible for regulating cell specific expression of the m4 gene. In collaboration

with others in the group, gel mobility shift assays have been employed to identify genomic elements in the m4 promoter that are capable of recruiting DNA binding proteins.

I show that the m4 gene is under the regulation of an RE1/NRSE which mediates repression on non-neuronal cells and a sub-population of neuronal m4 non-expressing cell lines. A GC box like sequence is also important in the regulation of m4 in a sub-population of neuronal m4 expressing cells. Further, I show that the m4 gene is regulated by two distinct independently regulated promoter complexes and that the combination of the outputs from the two promoters defines the m4 gene expression profile.

RESULTS/CONCLUSIONS

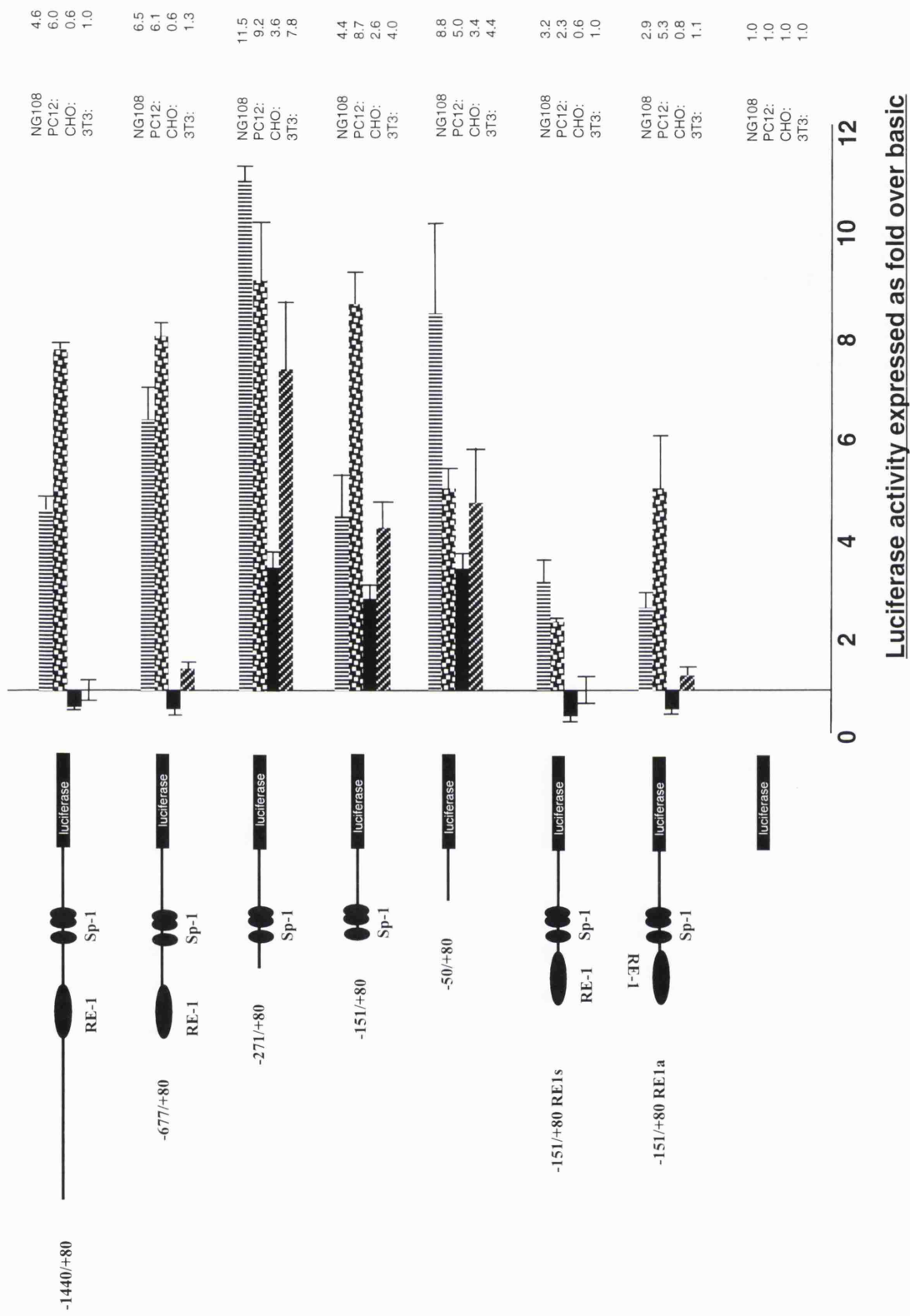
Initially, transient transfection analysis of m4 promoter constructs fused to luciferase coding sequence in pGL3LC were performed in two classes of cell lines i) m4 expressing neuronal (PC12 and NG108-15). ii) m4 non-expressing non-neuronal cells (3T3 and CHO)

The data are summarised in figure 1. The two larger constructs, pGL3LC -1440/+80 and pGL3LC -677/+80 are both capable of driving expression in the two m4 expressing cell lines but are silent in the non-expressing cell lines. Deletion of the region of the m4 promoter between -677 and -271, to give pGL3LC -271/+80 resulted in constitutive expression in all four cell lines, suggesting that there is a silencing element within this region. Sequence analysis of this region identified a 28 bp sequence with homology to a RE1/NRSE repressor element that has been implicated in repression of other neuronal specific genes such as the sodium type II channel gene (Maue *et al.*,1990), the SCG 10 gene (Mori *et al.*,1990), the dopamine β -hydroxylase gene (Ishiguro *et*

FIGURE 1. Expression of luciferase reporter constructs in transient transfection of NG108, PC12, CHO and 3T3 cell lines.

Transfections were performed in 24X15mm well trays using Qiagen column purified DNA and lipofectamine (Life Technologies Inc). 125ng of each m4 plasmid and 125ng of the β -galactosidase expressing CMV-GAL plasmid were incubated with 1.25 μ l lipofectamine in 50 μ l optiMEM for 30 minutes at room temperature. A further 150 μ l optiMEM was added and the whole 200 μ l added to a well of cells prewashed with PBS. After 24 hours at 37 $^{\circ}$ C the cells were washed and fed with 1ml of medium. Cells were harvested 24-48 hours post feeding and analysed using the Promega Luciferase Assay System in a Turner TD-20e luminometer.

All data are Luciferase values normalised to β -galactosidase expression driven by cotransfected pCMV β assayed using ONPG substrate as described. Numbers represent activity as fold over pGL3LC. Data represent the mean of three individual experiments, each performed in triplicate. Standard error is depicted by the error bars.



al.,1993) the Na⁺/K⁺-ATPase α 3 subunit (Pathak *et al.*,1994) and the synapsin I gene (Howland *et al.*,1991; Li *et al.*,1993; Schoch *et al.*,1996). The proximal m4 promoter appears to be constitutively active implicating the ability of ubiquitous factors to bind to this region and activate transcription. To ascertain whether the RE1/NRSE homologous sequence was indeed responsible for repression of m4 expression in these cell lines, two constructs were made that contained 28bp of promoter sequence corresponding to nucleotides -574 to -550 ligated immediately upstream of the proximal promoter region, (plasmids pGL3LC-151/+80 RE1s and pGL3LC-151/+80 RE1a). Both of these plasmids, when transfected into cell lines show repression of reporter activity in 3T3 and CHO cells to basal levels comparable with the levels obtained with pGL3LC-1440/+80. pGL3LC-151/+80 RE1s and pGL3LC-151/+80 RE1a both show reporter expression in NG108 and PC12 cells although the levels appear slightly reduced when compared to reporter activity driven by pGL3LC-151/+80. The reason for this effect is not completely clear, although it may reflect the presence of low levels of endogenous REST found in this cell line (Bessis *et al.*,1997).

The RE1/NRSE element was originally identified in the type II Na⁺ channel and SCG10 promoters and was shown to be responsible for silencing gene expression in non-neural cells. Since then DNA data base analysis has identified this element in 18 genes, most of which are selectively expressed in neural tissue. The sequence shows very high homology in various genes and is well conserved in species as diverse as human and chicken (figure 2). However, functional characterisation of this element has only been demonstrated for promoters of the type II Na⁺ channel, SCG10, synapsin 1, dopamine β -hydroxylase and Na⁺K⁺-ATPase α 3 subunit genes. In the case of the human synapsin I gene,

Schoch et al. dissected the promoter and concluded that the REST/NRSF binding motif was solely responsible for the neural specific expression of this gene (Schoch *et al.*,1996). In addition, a transgenic analysis of the RE1/NRSE site has been reported for one gene, the Na,K-ATPase α 3 subunit, and, in this case, 210bp of the sequence 5' to the transcription initiation site drove tissue-specific expression. Mutation of the RE1/NRSE site within this region, resulted in ectopic expression of the reporter gene in all non-neuronal tissues tested. Subsequently however, work carried out by Schoenherr *et al* has shown that this particular mutation of RE1 resulted in the formation of a new element able to recruit what appears to be an activator (Schoenherr *et al*, 1996). I demonstrate that deletion of this RE1 element in the m4 gene leads to expression in both non-neural and neural cells. Furthermore, direct juxtaposition of the RE1 element (in either orientation) to the constitutively active promoter re-establishes silencing of the gene in non-expressing cells. Gel mobility shift assays, (Wood *et al*,1997; Wood *et al.*,1996), showed that the RE1/NRSE sequence in the m4 promoter bound to a protein present in non-expressing cells but not in m4 expressing cells consistent with the notion that this sequence recruits a transcriptional repressor.

FIGURE 2. Comparison of the RE1/NRSE sequences from the m4 and other neural specific genes where this region has been identified as being functionally important in gene regulation.

(+) and (-) represents sequence found in the sense (+) and (-) orientation with respect to the m4 promoter. Data were taken from references cited in the 'Introduction'.

Genes such as the type II Na⁺ channel, SCG10 and synapsin 1, in which the RE1 silencing element has functional activity are all pan-neuronal genes that are widely expressed in most neuronal cells. In contrast, the m4 gene is expressed predominantly only in a subset of telencephalic neurones (Buckley *et al.*,1988) and autonomic neurones (Hassal *et al.*,1993; Brown *et al.*,1995). Since REST/NRSF is not expressed in any differentiated neuronal cells in the CNS (Chong *et al.*,1995; Schoenherr and Anderson,1995) it follows that there must be a set of neuronal cells which do not express REST/NRSF and which do not express the m4 gene (and also cells that express REST and are m4 positive). Therefore the m4 gene must be under the control of other regulatory factors in addition to REST/NRSF. Two possibilities arise: (1) Another repressor protein or set of proteins is responsible for silencing m4 expression in those neurons that do not express m4; (2) *In vivo* the m4 proximal promoter may not be constitutively active in neuronal cells and that specific activators would drive expression in neuronal subpopulations. The latter possibility infers that *in vitro* transcriptional regulation may not accurately reflect gene regulation *in vivo*. Many examples exist of these disparities, such as the nicotinic receptor α subunit gene where a proximal promoter drives myotube specific expression *in vitro* but fails to recapitulate specific expression in transgenic mice (Klarsfeld *et al.*,1991).

Closer analysis of promoter activity shows that in PC12 cells, there is an approximate 50% reduction in reporter gene activity upon removal of the region between -151 and -50. This region contains a single GC box and also a GC box dimer sequence. This raises the possibility that Sp1 maybe involved in defining the extent of m4 expression in PC12

cells although proof of this hypothesis will have to await gel shift and *in-vivo* footprint analysis. Interestingly, the GC box dimer sequence does not seem to be required for activity of the de-repressed m4 promoter in 3T3 or CHO cells. This is intriguing because gel shifts with -151/+80 as probe shows that in CHO cells at least, Sp1 is capable of binding to the Sp1 dimer site (see figure 4 page 14223 of Wood *et al*, 1996). Band "a" in figure 4, page 14223 of Wood *et al*, 1996 corresponds to the lowest mobility band seen in Sp1 gel shifts in a number of reports e.g. band "A1" in figure 5 of Vinals *et al*, 1997 (Vinals *et al.*,1997) (which itself resolves into bands "A1" and "A1'" at higher resolution and probably represent two states of phosphorylation of Sp1 as described (Jackson *et al.*,1990)). Band "a" from Wood *et al*,1996 also corresponds to the closely migrating bands "1" and "2" of figure 3, 4, 5 and 6 of Vindevoghel *et al*, 1997 (Vindevoghel *et al.*,1997). It is apparent that the signal intensity of the gel shifts in Wood *et al* 1996 are fainter than the two references cited. Due to this, the fainter high mobility bands typically expected of an Sp1 gel shift are not easy to discern in the published figure.

Further, the gel shift performed in the presence of anti-Sp1 polyclonal antibody (obtained from Santa Cruz) in Wood *et al* 1996 (figure 4 page 14223) shows a large diminution of band "a" and a faint appearance of a higher mobility band (labelled +Ab in figure 4 page 14223). The signal intensity of the supershifted band is less than that for band "a" because the antibody is polyclonal and thus gives rise to a population of antibody/Sp1 complexes with differing mobilities. This phenomenon has also been observed by other researchers. For example, a smeared low intensity supershift with Sp1 antibody is seen in figure 3C of Marin *et al*, 1997 (Marin *et al.*,1997) and further, complete

absence of the primary Sp1 shift is NOT seen in Marin *et al* 1997 as is it not seen in Wood *et al* 1996. The above comparisons suggest that the bands seen in gel shifts performed with the -151/+80 region of the m4 promoter by Dr I.C Wood and reported in Wood *et al* 1996, do have correlates with bands seen in gel shift experiments performed by others. It is likely then that band a at least can be explained by Sp1 binding to the GC dimer.

Also, a canonical GC box/Sp1 binding site was used in competition gel shift assays with the -151/+80 region of the m4 promoter (see lanes 6 and 7 of figure 4 page 14223 Wood *et al* 1996). These show competition with band "a" but not "d","e", or "f". Bands "d","e" and "f" are specific because they are competed with excess cold -151/+80 sequence. Therefore they most likely represent proteins other than Sp1 which bind this region of the m4 promoter.

So, in summary, my deletion data and the gel shift data reported in Wood *et al* 1996, support a model in which Sp1, though able to bind the Sp1 sequences in the m4 proximal sequence, **fails to** upregulate the m4 promoter in CHO cells and is therefore **not** involved with the regulation of m4 de-repressed promoter activity in this cell line. Gel shifts with -151/+80 produces a number of other bands, all of which are attributable to proteins binding the GC dimer sequence.

It appears then, that the combined action of the RE1 sequence and the GC dimer on the m4 constitutively active core promoter complex contribute to the expression profile of m4 in the four cell lines analysed.

I next sought to define the mechanisms which restrict m4 expression to those subsets of neuronal cells in the nervous system where m4 is expressed. NBOK1 cells express REST and do not express

m4. Transient transfection analysis in this cell line with m4 deletion constructs shows that 1.5Kb of proximal upstream promoter sequence is silent in these cells (figure 3a). Deletion of 400bp from -677/+80 to -271/+80 results in nearly 5 fold increase in promoter activity suggesting removal of a repressor element. Juxtaposition of RE1 sequence to the minimal -151/+80 fragment re-establishes silencing (figure 3b). This suggests that in NBOK1 cells, repression of m4 expression is mediated at least in part by the RE1 element acting on a constitutively active core promoter. Also, removal of a fragment containing the GC box and GC box dimer causes a 3 fold reduction in promoter activity (compare -50/+80 with -151/+80). This raises the possibility that Sp1 may be involved in governing the expression level of the m4 promoter in this cell line. However, without Sp1 binding data in the form of gel shifts and footprints, it is equally likely that non-Sp1 proteins mediate this activity.

Analysis of the cell line Neuro-2A (N2A) was very surprising. N2A cells express REST at levels *ca* 12 fold less than 3T3 fibroblasts (Lonnerberg *et al.*,1996) and do not express m4. Transient transfection analysis showed that all m4 promoter containing constructs expressed approximately 2 fold over basic (figure 3a). This suggests that an essential repressor element is missing in the deletion constructs used and that REST is not responsible for the lack of m4 expression in this cell line. Two possibilities arise:i) REST may be present at too low a concentration to mediate efficient binding to the m4 RE1 sequence and repress the promoter. ii) the m4 core complex recruited in N2A cells may be immune to the repressing effect of REST. If the former is true then raising the nuclear concentration of REST in N2A cells should re-establish repression. Titrated transient co-transfections of pMT-HZ4 (a

plasmid expressing the 1.8Kb N-terminal region of REST encompassing the N-terminal repression domain and DNA binding domain) with m4 reporter constructs pGL3lc-677/+80 (contains RE1) and -151/+80 (lacking the RE1 sequence) showed no RE1 dependent repression (figure 4). This argues against the m4 promoter recruiting a REST repressible core complex in N2A cells that is active due to limiting amounts of REST in the cell. The intriguing implication then is that the core complex recruited to the m4 promoter in N2A cells is functionally different from that recruited in the REST expressing cell line 3T3. That a given promoter can recruit distinct core/TFIID complexes in a cell type specific manner has been suggested by Tamuara *et al* for the mouse myelin basic protein gene (Tamuara *et al.*,1990). *In vitro* they showed that the MBP core promoter was selectively transcribed by brain derived TFIID and not liver derived TFIID yet both complexes drove expression from the adenovirus major late promoter.

The TFIID complexes can vary according to the TAF complement associated with the complex or the actual isotype of TBP. The work of Chen and Struhl (Chen and Struhl,1988) on the yeast HIS3 promoter suggests that eukaryotic cells possess a number of different TBP isotypes and that each can confer specific activities to core promoters. The ability to select core complexes in this manner would allow the gene to come under the control of different sets of transcription factors depending on the cell type. This is of obvious advantage when one considers that a gene under the control of a given transcription factor in one cell may not want to be under that same control in a different cell type where this transcription factor is also found. The recruitment of distinct complexes to a given promoter thus results in a very large

FIGURE 3. Expression of luciferase reporter constructs in transient transfection of NBOK1 and Neuro-2a cell lines.

a) Serial deletion constructs of the m4 promoter transiently transfected into NBOK1 and Neuro-2a cells.

b) minimal constructs RE1s-151/+80 and RE1a-151/+80 transiently transfected into NBOK1 cells.

Transfections were performed in 24X15mm well trays using Qiagen column purified DNA and lipofectamine (Life Technologies Inc). 125ng of each m4 plasmid and 125ng of the β -galactosidase expressing CMV-GAL plasmid were incubated with 1.25 μ l lipofectamine in 50 μ l optiMEM for 30 minutes at room temperature. A further 150 μ l optiMEM was added and the whole 200 μ l added to a well of cells prewashed with PBS. After 24 hours at 37°C the cells were washed and fed with 1ml of medium. Cells were harvested 24-48 hours post feeding and analysed using the Promega Luciferase Assay System in a Turner TD-20e luminometer.

All data are luciferase values normalised to β -galactosidase expression driven by cotransfected pCMV β assayed using ONPG substrate as described. Numbers represent activity as fold over pGL3LC. Data represent the mean of three individual experiments, each performed in triplicate. Standard error is depicted by the error bars.

Figure a

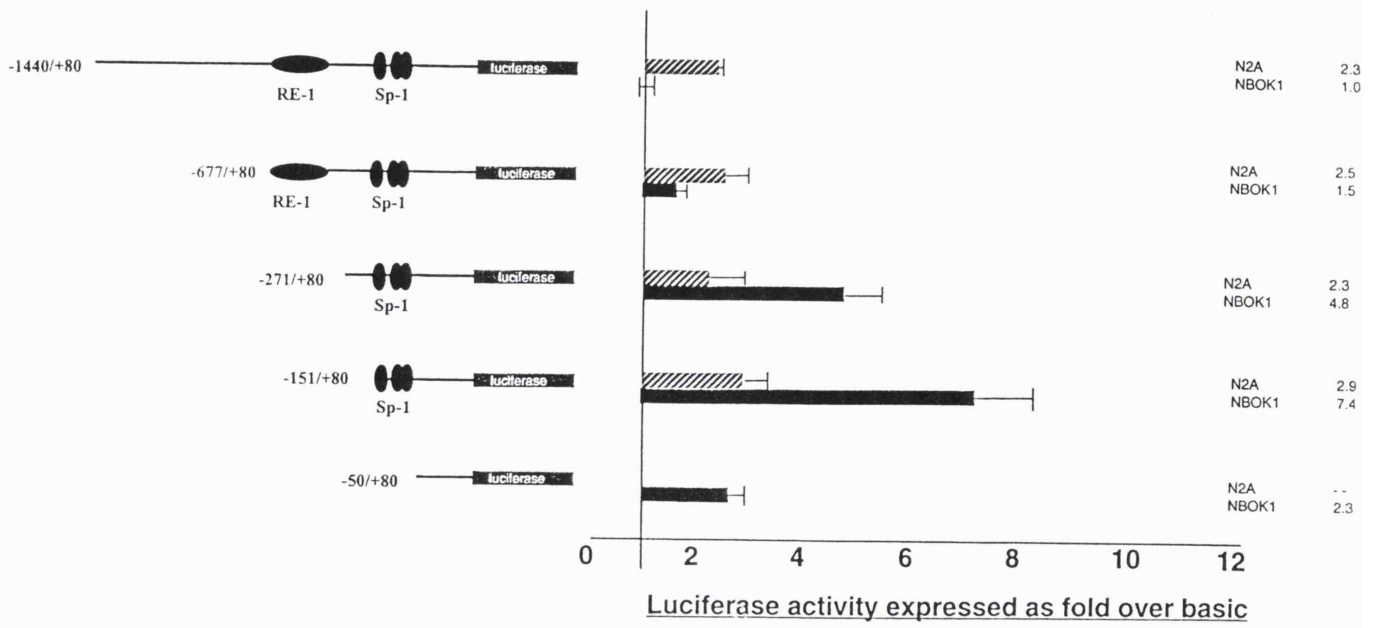


Figure b

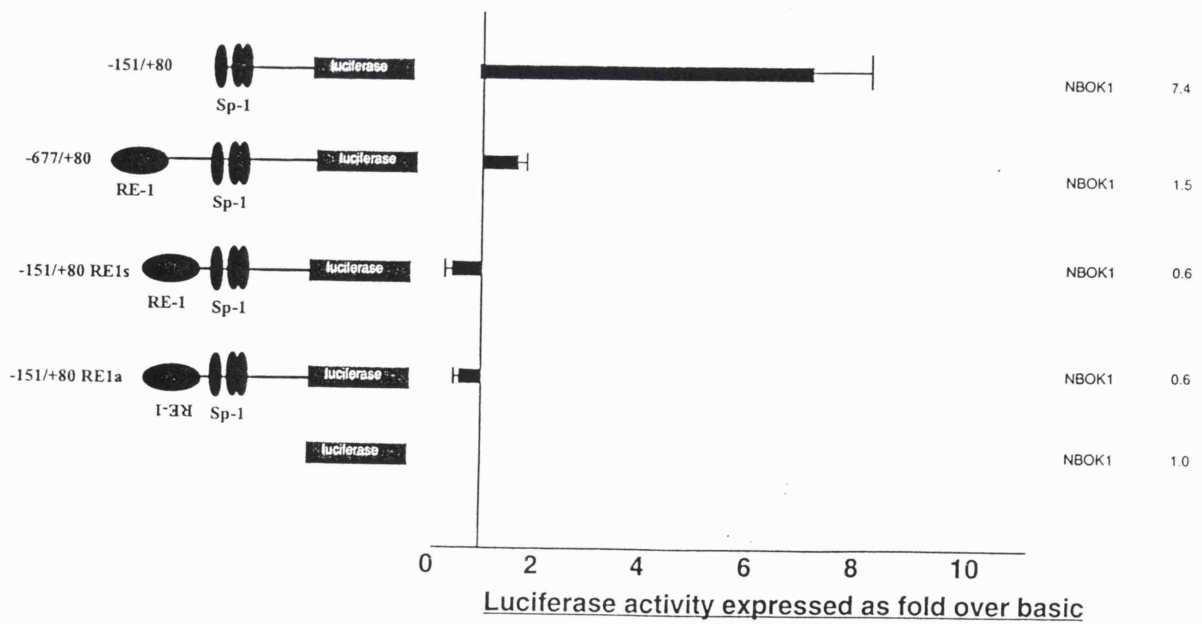
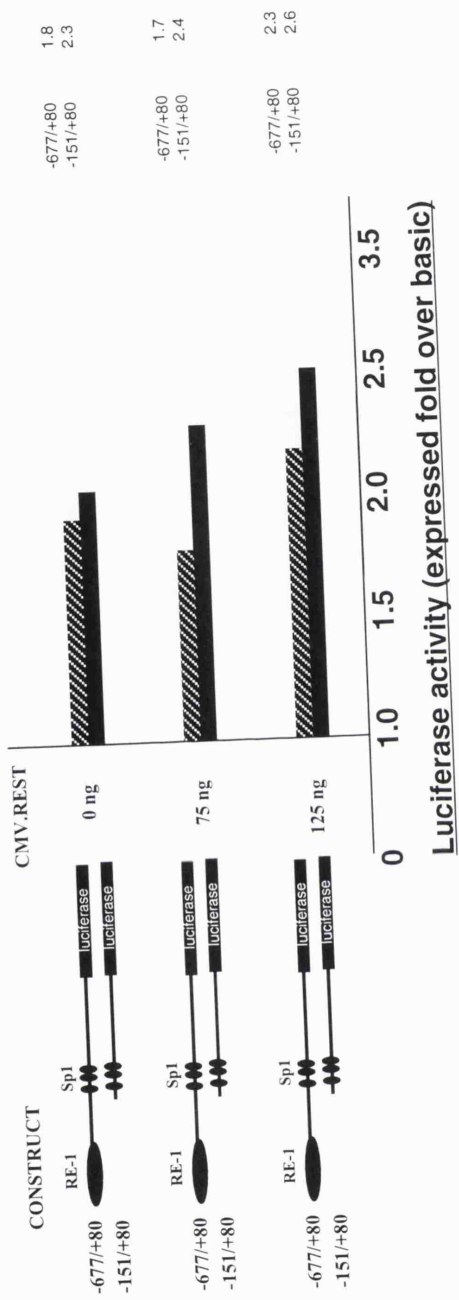


FIGURE 4. Expression of luciferase reporter constructs in Neuro-2a cell lines in the presence of recombinant REST.

The m4 promoter is not repressible by increased REST levels in Neuro-2a cell.

Transfections were performed in 24X15mm well trays using Qiagen column purified DNA and lipofectamine (Life Technologies Inc). 125ng of reporter plasmid was co-transfected into Neuro-2a cells with 75ng and 125ng of CMV-REST and 1ng CMV-Renilla were incubated with 1.25 μ l lipofectamine in 50 μ l optiMEM for 30 minutes at room temperature. A further 150 μ l optiMEM was added and the whole 200 μ l added to a well of cells prewashed with PBS. After 24 hours at 37 $^{\circ}$ C the cells were washed and fed with 1ml of medium. Cells were harvested 24-48 hours post feeding and analysed using the Promega Dual Luciferase Assay System in a Turner TD-20e luminometer.

All data are luciferase values normalised to Renilla expression driven by cotransfected CMV-Renilla. Numbers represent activity as fold over pGL3LC. Data represent the average of two individual experiments, each performed in triplicate. The error was less than 20%.



increase in the diversity of expression patterns afforded to a set of genes under the control of a limited number of transcription factors.

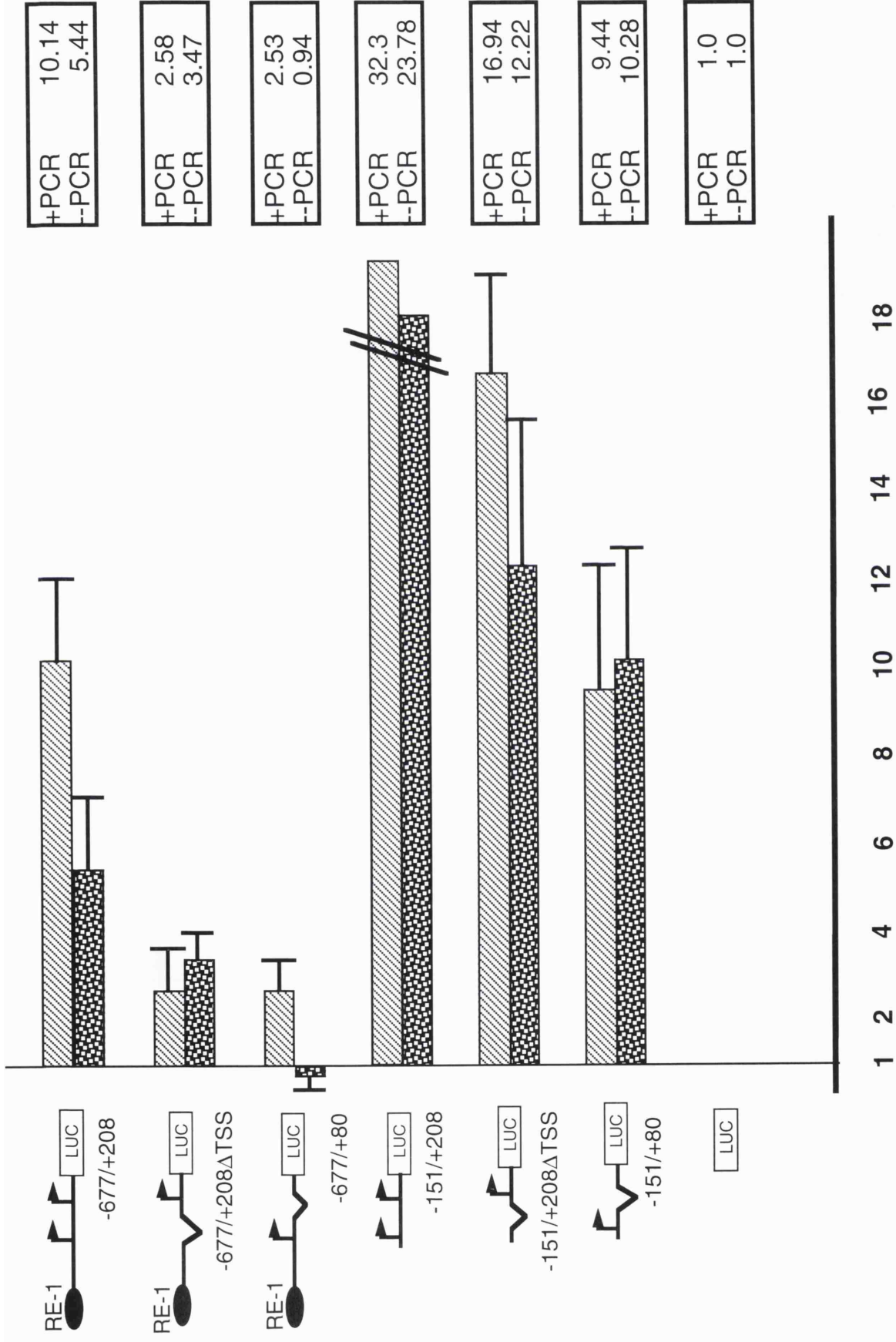
REGULATION OF DUAL PROMOTERS IN 3T3 CELLS

As mentioned previously, Mieda *et al* (Mieda *et al.*,1996) defined two start sites separated by 1bp ca 280bp down-stream of the one defined by our analysis. Their experiments showed that constructs encompassing both upstream and downstream sites behaved in a qualitatively similar manner in transient transfections of NG108-15 and L6 cells. To assess the behaviour of the two start sites/promoters in our model cell lines I performed transient transfection assays with constructs containing either or both start sites with and without the RE1 sequence into the m4 non-expressing fibroblast cell line 3T3. I then looked at the effects of quenching out REST with RE1 containing PCR products on reporter gene expression driven from the various start site combinations. The results are summarised in figure 5.

Strikingly, in 3T3 cells, -677/+208 gave potent expression despite -677/+80 being silent (figure 5). This suggests that the down stream tsp (tsp-d) is active in these cells. To assess the role of REST and the RE1 element on the 2 tsps, a PCR product containing 3 RE1 sites was co-transfected along with the reporter to quench REST. This resulted in a 1.9 fold increase in reporter activity. Removal of the upstream start site (tsp-u) to give -677/+208 Δ TSS resulted in a 40% reduction suggesting that the tsp-d contributes a slightly larger fraction of the overall reporter signal. Quenching of REST with RE1 sites showed no increase of signal with -677/+208 Δ TSS suggesting that REST does not repress the down-stream complex. In fact a slight loss of signal is observed which may be the result of 1) REST directly activating tsp-d though not by a very significant degree (1.4 fold) or 2) REST repressing a tsp-d specific repressor thus quenching results in expression of this repressor and

FIGURE 5. Analysis of 3T3 cells co-transfected with m4 dual promoter constructs and RE1 containing PCR products.

'+PCR' represents cells co-transfected with reporter and RE1 containing PCR product generated as described under 'Materials and Methods'. '-PCR' represents cells co-transfected with reporter and PCR product lacking RE1 sites. Transfections were performed in 24X15mm well trays using Qiagen column purified DNA and lipofectamine (Life Technologies Inc). 125ng reporter plasmid, 1ng Renilla expressing CMV-Renilla plasmid and 125ng gel purified/electro-eluted and precipitated PCR product were incubated with 1.25 μ l lipofectamine in 50 μ l optimem for 30 minutes at room temperature. A further 150 μ l optimem was added and the whole 200 μ l added to a well of cells prewashed with PBS. After 24 hours at 37°C the cells were washed and fed with 1ml of medium. Cells were harvested 24-48 hours post feeding and analysed using the Promega Luciferase Assay System in a Turner TD-20e luminometer. All data are normalised to Renilla expression driven by cotransfected CMV.renilla. Numbers represent promoter activity as fold over pGL3LC, as defined by pGL3LC co-transfected with or without RE1 site containing PCR product. Data represents the mean of three individual experiments, each performed in triplicate. Standard error is depicted by the error bars.



Luciferase activity expressed as fold over basic

down regulation of *tsp-d*. That REST may act as an activator, which is the assumption implicit in the former possibility, has been suggested by elegant experiments reported by Bessis *et al* (Bessis *et al.*,1997). However, the mode of REST mediated activation described there suggests a mechanism analogous to the lambda repressor i.e. REST must be juxtaposed next to the core promoter complex, placing the RE1 site further away fails to show activation. In the constructs used here, the RE1 site is placed away from the core complex and thus activation must occur via a different mechanism. If REST represses a repressor, then the cognate element of this unidentified repressor must lie within -677/+208.

As expected REST quenching results in potent up-regulation of *tsp-u* (2.7 fold) i.e. -677/+80. Curiously, all constructs lacking an RE1 site expressed at higher levels than all the corresponding RE1 containing constructs co-transfected with RE1 containing PCR product. If RE1 is solely responsible for silencing of *tsp-u* and REST quenching is efficient then -677/+80(+PCR) should express at the same level as -151/+80. The fact that the former construct expresses at lower levels than the latter suggests that i) REST is not solely responsible for repression of *tsp-u* or ii) other element(s) contribute to silencing of *tsp-u*. The latter possibility is unlikely because juxtaposition of an RE1 site immediately upstream of -151/+80 results in silencing of expression to basal levels. Therefore, REST quenching probably is not complete by use of RE1 containing PCR product. *Tsp-d* however, is not repressed by REST as judged by the comparable activities of the fact -677/+208 Δ TSS+PCR and -677/+208 Δ TSS-PCR. Therefore the increased activity of -677/+208 Δ TSS+PCR over -151/+208 Δ TSS suggests that there is a repressor acting on *tsp-d* which is present between -677 and -151.

The implication of the above observations is that the m4 gene is transcribed by 2 promoters which are differentially regulated by REST. Further, repressor activities must exist to silence *tsp-d* in 3T3 cells. Any elements required for this silencing are i) not in the largest construct used in our deletion analysis ii) are within the constructs used in the assays but lack the cognate repressing factors in 3T3 cells or iii) are unable to function in transient transfection experiments. As such it is possible that the repressor activities implicated between -677 and -151 in the previous discussion may *in vivo* be responsible for silencing *tsp-d* but cannot function adequately in transient transfection assays. However, proof of this hypothesis is required.

In the absence of an RE1 element, quenching gave some upregulation (1.4 fold) of *tsp-d* but no upregulation of *tsp-u*. This suggests that there is a RE1 independent mechanism of REST repression working on *tsp-d* though the effect is much less marked. Interestingly Mieda *et al* (Mieda *et al.*,1996) also reported an RE1 independent repression of their *tsp-d* containing constructs and overcame this by exchanging the luciferase reporter gene for CAT but failed to address the nature of this mechanism. Analysis of the luciferase coding sequence shows the presence of an X-box like element (830-GAATGTT-835; X-box sequence= GAATGTC). The X-box element was originally located in the promoter of the MHC-II gene, DPA, and found to bind the C-terminal Zn finger of REST(Schollet *et al.*,1996). It is possible that REST binds to this element to mediate RE1 site independent repression. It is interesting to speculate then that because RE1 independent repression is only observed with *tsp-d* then REST may repress *tsp-d* and *tsp-u* differentially depending on whether it binds RE1 or the X-box like element. However, proof of this hypothesis is required.

An interesting difference exists between the expression profile of RE1 containing m4 constructs in 3T3 cells and the behaviour of these constructs in the myoblast cell line L6 as reported by Mieda et al. In 3T3 cells, the RE1 containing fragments -1044/+80 and -677/+80 are silent whereas -677/+208 shows potent expression. However, the equivalent Mieda construct pGL2-P1074 (corresponds to -790/+208) is silent in the cell line L6. It may be the case that the 113bp sequence found in pGL2-P1074 contains a repressor element that is absent in -677/+208. If this is the case then addition of this sequence to -151/+208 Δ TSS should result in silencing of reporter expression if 3T3 cells also express this repressor. However, gel shift experiments with the m4 region -1044 to -677 fail to show binding in 3T3 extracts (personal communication, I.C.Wood).

Another possibility is that a repressor element exists in both pGL2-P1074 and -677/+208 but the factor is only found in L6 cells. The implication is, as mentioned above, 3T3 cells possess an unidentified repressor that binds an element that is i) missing in -677/+208 or ii) is unable to operate in a plasmid borne system.

Alternatively, the core-complex recruited to tsp-d (assuming that in L6 cells, tsp-u is silenced by REST as in 3T3 cells) may be different in L6 cells as compared to that recruited in 3T3 such that REST is able to mediate repression of tsp-d in the former but not the latter cell type. Thus in L6 cells, REST would be responsible for repression of both promoters. If this hypothesis transpires to be correct then an interesting question would be how does a single copy of an element mediate repression of 2 promoters simultaneously? The cognate factor, in this case REST, can presumably only bind to one complex at any one time and thus leave the other complex free from repression. A

mechanism can be envisaged which allows REST to impose silencing over a large region of DNA by 'seeding' the formation of repressive chromatin structure. This would allow many promoters to be silenced simultaneously. The implications for such a mechanism with respect to REST co-repressors and transient transfection assays are elaborated in 'Discussion'.

CHAPTER 4.

ISOLATION AND CHARACTERISATION OF DNA BINDING PROTEINS

CHAPTER 4

ISOLATION AND CHARACTERISATION OF DNA BINDING PROTEINS

INTRODUCTION

THE 1-HYBRID SYSTEM

The 1 Hybrid reporter (Wang and Reed,1993) system is a means of carrying out an *in vivo* screen for DNA binding proteins. The assay makes use of 2 observations: i) Many yeast promoters have two distinct sequence domains - the minimal promoter or Upstream Essential Sequence (UES) which confers basal promoter activity, and the Upstream Activator Sequence (UAS) which binds transactivators and allows up-regulation of the UES. Further, the UAS can be removed and replaced with any other target sequence such that the minimal promoter will now be regulated by transactivators that can bind the new UAS. ii) Transcription factors (TFs) such as GAL4 have distinct DNA binding domains and activation domains such that the DNA binding domain of one TF can be joined to the activation domain of another TF (see Ptashne,1997 for review). For example, if the activation domain of the yeast transcription factor gal4p is fused to the DNA binding domain of the *E.coli* factor LexA, the hybrid protein will now activate the GAL1 promoter only if the lexA Operator sequence is present in place of the GAL4 binding sequence (UAS) in the GAL1 minimal promoter.

In a 1 hybrid screen, a target sequence is cloned in place of the UAS in a yeast promoter e.g. GAL1 and the new hybrid promoter is used to drive a selectable marker gene such as HIS3. The yeast strain containing this construct is then transformed with an expression library containing cDNAs fused to the GAL4 activation domain. In those cells

where the GAL4 activation domain is fused to a protein which binds the target sequence, the GAL1 minimal promoter will be upregulated and express the selectable marker conferring growth on that cell on selective media. The library plasmid is then extracted from the resulting colony and amplified in *E.coli* to give the sequence of the DNA binding protein.

Deletion analysis and EMSA experiments showed two *cis*-elements to be important in the regulation of the m4 promoter: the RE1 site and the Sp1 dimer at position -67. Both of these sequences were chosen as baits to screen various libraries for transcription factors that could bind these elements and thus potentially be important in the regulation of the m4 gene.

Since REST/NRSF is not expressed in any differentiated neuronal cells in the CNS (Chong *et al.*,1995) it follows that there must be a set of neuronal cells which do not express REST/NRSF and which do not express the m4 gene. Therefore the m4 gene must be under the control of other regulatory factors in addition to REST/NRSF. The implication is that another repressor protein or set of proteins is responsible for silencing m4 expression in those neurons that do not express m4. If a subset of these proteins act via the RE1 element then they could be isolated by using the m4 RE1 sequence as bait in a 1-Hybrid screen.

The distinct advantage of cloning any novel RE1 binding factors by 1-hybrid approaches over cloning by homology is that the former makes no assumptions about the structure of the novel factor, only that it binds the bait sequence. Cloning novel RE1 binding factors by PCR using degenerate primers to the Zn fingers assumes that all RE1 binding

proteins are of this Zn finger class. There is no *a priori* reason why this should be the case.

Three cDNA libraries were screened using the above bait: adult rat brain, E17 whole mouse embryo, and E11 whole mouse embryo derived cDNA fused to the GAL4 activation domain. Two different developmental ages were chosen because REST itself is known to be expressed in neuronal precursors during embryogenesis. Thus if a family of RE1 binding proteins exist then they may be expressed during various stages of embryogenesis but probably with overlapping yet distinct spatial patterns. Clones that were isolated using the 1-hybrid protocol are described and the implications they pose for RE1 dependent regulation of the m4 gene are discussed. Subsequent analysis of RE1 sequences and their role in the differential regulation of RE1 containing genes via their interaction with REST are described. Finally, I describe some pilot experiments aimed at modifying the 1-hybrid screen to eliminate yeast colonies that arise due to mutation events independent of true 1-hybrid interactions.

RESULTS AND CONCLUSIONS

SCREEN FOR RE1 BINDING PROTEINS

Approximately 2×10^7 yeast transformants were screened with the adult rat brain library containing 2×10^6 independent clones. 50 robust colonies were obtained on SD-Trp-Leu-His media. However, isolation of the library plasmid from each colony and subsequent retransformation back into the host strain failed to recapitulate histidine autotrophy. This suggests that GAL1 activating mutations were responsible for HIS3 (and thus histidine autotrophy) in the original screen.

8×10^6 yeast transformants were screened with the E17 whole mouse library which had a similar complexity to the adult rat brain library. Fourteen robust colonies were obtained on triple drop out media. Library plasmids were isolated and re-transformed into the host strain. One candidate, 2-TB5-1 conferred histidine autotrophy. This candidate was chosen for further characterisation. 2-TB5-1 was transformed into a host strain containing the reporter plasmid pBM2389 without any RE1 sequence as bait. However, this failed to restore histidine autotrophy. The implication is that GAL1 activation is bait specific.

Sequencing of the entire 2-TB5-1 insert showed no homology to anything in the databases. Also we found no discernible DNA binding domain. Further, sequencing failed to show any open reading frame (ORF) in frame with the GAL4 activation domain. The largest ORF extended from position 2017 to 1169 in the antisense direction encoding a putative protein of Mr=32Kd (see figures 6a). 2-TB5-1 was then transformed into yeast containing GAL1-LacZ reporter plasmids with single, double and triple m4 RE1 sequences as bait. No activation over control (GAL4 AD without DNA binding activity) was seen with 1 site but 60% activation was seen with 2 and 3 sites (see figure 6b). The failure to see activation with a single site yet the same fold activation with 2 and 3 sites is consistent with the candidate recognising a junction between the RE1 sequences rather than the RE1 element itself.

Because of the failure to find i) an ORF in frame with GAL4AD, ii) any discernible DNA binding domain, iii) activation with a single RE1 element we decided to invest no further effort in the characterisation of this clone. Any one of the above reasons by themselves would not be sufficient reason to terminate the further characterisation of a

FIGURE 6 Open Reading Frame and RE1 binding analysis of 2TB5-1.

a) Open reading frames predicted by sequence analysis of 1-hybrid candidate 2TB5-1. Lanes 1,2 and 3 are sense; lanes, 4, 5 and 6 are antisense.

b) β -GAL activity derived from CYC1-LacZ reporter plasmids containing 1 RE1 element or a tandem array of 2 or 3 RE1 elements derived from the rat Sodium Type II promoter. Cells bearing the reporter plasmid expressed either the 1-hybrid candidate 2TB5-1, pACT2.DomNeg which expresses the gal4 activation domain fused to the RE1 DNA binding domain derived from mouse REST/NRSF or pACT which expresses the gal4 activation domain by itself.

Values are A_{420} measurements normalised to cell density and time of reaction expressed as fold over pACT2 as stated in 'Materials and Methods'. All values are the average of 3 independent experiments, each performed in triplicate using three independent yeast colonies. All errors were less than 15%.

Figure A

PREDICTED OPEN READING FRAMES GREATER THAN 25 AMINO ACIDS LONG IN 2TB5-1

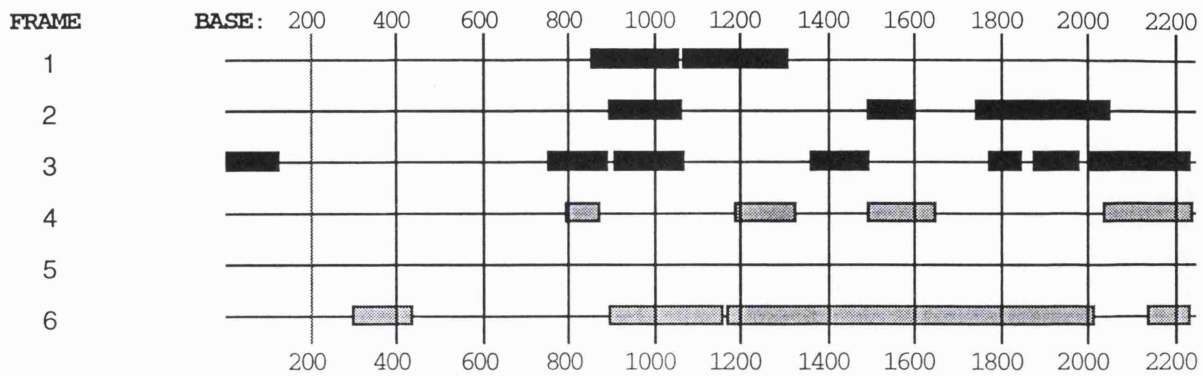


Figure B

REST DNA BINDING DOMAIN EXPRESSION CONSTRUCT	RE1 ELEMENT	β -GAL ACTIVITY
pACT2.DomNeg	0XNaIIRE1	0.8
2-TB5-1	0XNaIIRE1	0.9
pACT2.DomNeg	1XNaIIRE1	6.5
2-TB5-1	1XNaIIRE1	0.9
pACT2.DomNeg	2XNaIIRE1	6.7
2-TB5-1	2XNaIIRE1	1.6
pACT2.DomNeg	3XNaIIRE1	6.4
2-TB5-1	3XNaIIRE1	1.3

candidate. However, together they suggested that we were not looking at a transcription factor involved with the RE1 mediated regulation of the m4 gene.

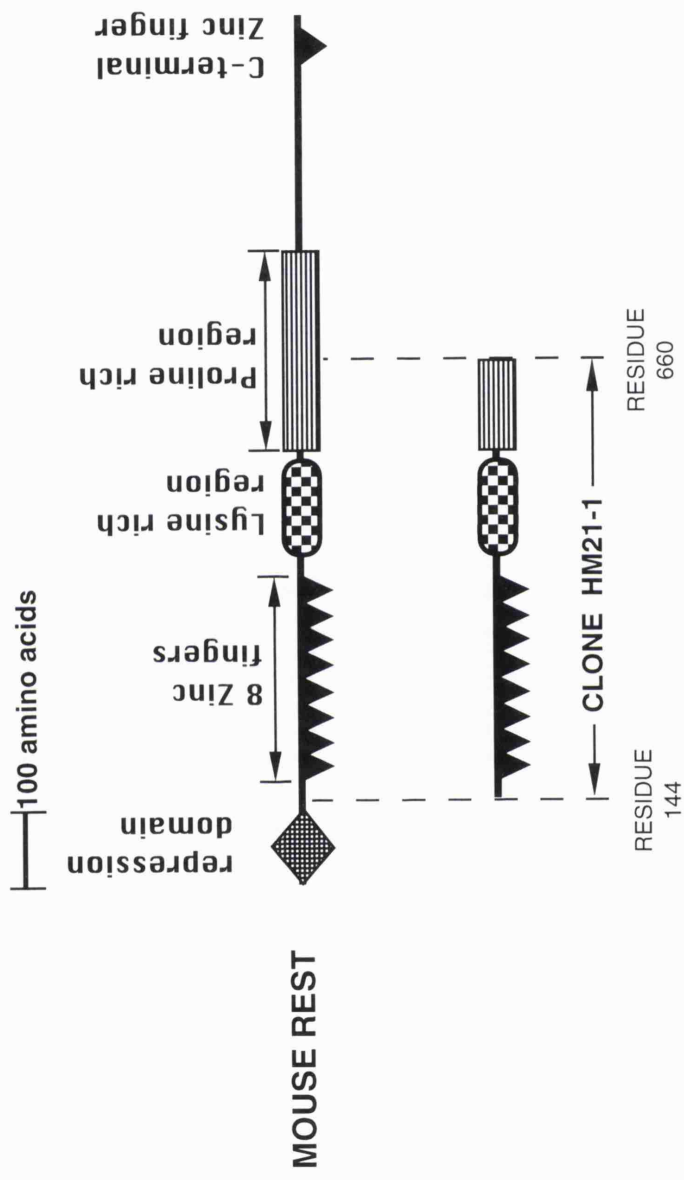
The third library screened was a E11 whole mouse derived cDNA library. Approximately 6×10^6 yeast transformants were screened using a library with a complexity of 2×10^6 independent clones. 20 robust colonies were obtained, library plasmid isolated and retransformed into the host strain. One candidate, HM21-1 conferred histidine autotrophy upon retransformation. Sequencing showed HM21-1 to encode amino acids 138 to 660 of mouse REST in frame with the GAL4 activation domain. A map of HM21-1 overlaid on mREST is shown in figure 7. As expected, GAL1-HIS3 activity was dependent on the presence of RE1 sequences as bait.

HM21-1 possessed the entire DNA binding domain (N-terminal 8 Zn fingers) and extends to the 5' end of the proline rich region (defined as starting at the Nco1 site at codon 633 and ending at codon 816). Interestingly, HM21-1 starts at codon 138, 65 residues downstream from the end of the N-terminal repression domain. It is possible that clones exist within the library, that extend the extra 414 bases to encompass the repression domain but fail to come through the screen due to the ability of the repression domain to repress the yeast GAL1 promoter. However, proof of this hypothesis needs confirming.

The library has not been screened exhaustively as judged by the fact that we have yet to isolate the same clone more than once. However, over 3×10^7 transformants have been screened using the RE1 sequence as bait and three libraries. We felt that further effort was not

FIGURE 7 1-hybrid candidate HM21-1 is a fragment of mouse REST.

Sequencing the ends of HM21-1 defined the domains of mREST encompassed by this clone. HM21-1 contained the DNA binding domain, lysine rich region and 60 residues of the proline rich region.



justified in isolating novel RE1 binding protein until we had direct proof that such proteins existed.

In the absence of proof that novel RE1 binding factors existed I decided to explore other mechanisms that would allow a minimal set of transcription elements and factors to regulate the maximum number of genes. Juxtaposition of m4RE1 sequences to the m4 proximal promoter (to give the construct pGL3LC-151/+80 RE1) resulted in repression of reporter gene expression in 3T3, CHO and NBOK1 cells. However down regulation of m4 promoter activity in this construct was also observed in PC12 cells. The work of Tapia-Ramirez (Tapia-Ramirez *et al.*,1997) and Bessis (Bessis *et al.*,1997) shows that PC12 cells express very low but discernible levels of REST. It is possible then that at this low concentration of REST, the m4 RE1 element at ca 677 bp away from the proximal promoter cannot mediate repression. At 150bp however, the low level of REST is more able to contact the repression machinery. If this is the case, then one has to explain why placing the NaII RE1 sequence at a similar distance from the NaII proximal promoter does not elicit repression in PC12 cells (Chong *et al.*,1995).

One possibility for the differences in the behaviour of the above 2 constructs in the presence of low amounts of REST is that the NaIIRE1 sequence has a lower affinity for REST than the m4RE1 sequence. In order to assess the difference in affinity of the 2 elements for REST I cloned a single copy of the m4RE1 and NaIIRE1 sequence upstream of CYC1-LacZ in pBM1933. The constructs were transformed into a yeast strain expressing high levels of a REST DNA binding domain/GAL4 activation domain fusion protein. Both constructs gave 32 to 34 fold activation of CYC1-LacZ as compared to a construct expressing just the GAL4 activation domain (figure 8). To ensure that the RE1 sites were

not saturated under these conditions, the strong ADH promoter driving fusion protein expression was removed. This ensured that fusion protein production was driven by weak cryptic activity derived from the backbone of the expression plasmid. Figure 9 shows the difference in expression levels of REST fragments driven by the ADH promoter and cryptic activity. Under these conditions, m4RE1 and NaIIRE1 gave 3.5 and 4 fold activation as compared to a construct expressing just the GAL4 activation domain. These results suggest that there is no significant difference in the affinities of the 2 elements for the DNA binding domain of REST.

That the core complex of the NaII promoter recruited in PC12 cells is repressible at all by REST is evident because over expression of REST in transient transfection studies shows that at high REST concentrations the NaII promoter is silenced (Chong *et al.*,1995). So the question remains as to how at low levels of REST, the m4 promoter is repressible and the NaII promoter is not yet both RE1 elements are of the same affinity and both core complexes are repressible at high concentrations of REST. It is possible that because the NaII core promoter is stronger than the m4 core promoter, the degree of repression at any given (non-saturating) repressor concentration is less for the NaII than m4 promoter. This relationship between fold repression and intrinsic promoter strength has been documented for repressors and promoters in the context of repressor distance (and thus local concentration) from core complex promoter (Saha *et al.*,1993).

The m4 gene is very localised in its expression within the adult nervous system. The NaII gene however has a very broad expression profile in the embryonic and adult nervous system. It follows then that there must be cells which express the NaII gene but not the m4 gene.

FIGURE 8. Analysis of REST binding to the m4 and NaII derived RE1 elements.

β -GAL activity derived from CYC1-LacZ reporter plasmids containing 1 RE1 element derived from either the rat Sodium Type II promoter or rat m4 promoter. Cells bearing the reporter plasmid contained pACT2.DomNeg (which expresses the gal4 activation domain fused to the RE1 DNA binding domain derived from mouse REST/NRSF), or pRS423.21-1 (which expresses very low levels of the gal4 activation domain fused to a region of REST/NRSF encompassing the DNA binding domain) or pACT (which expresses the gal4 activation domain by itself).

Yeast colonies were picked into 5mls of YPD and grown overnight to a cell density of 0.8-1.0, harvested and lysed using glass beads. β -galactosidase activity was assayed using the ONPG method as described. Values are A_{420} measurements normalised to cell density and time of reaction expressed as fold over pACT2 as stated in 'Materials and Methods'. All values are the average of 3 independent experiments, each performed in triplicate using three independent yeast colonies. All errors were less than 20%.

REST DNA BINDING DOMAIN EXPRESSION CONSTRUCT	RE1 ELEMENT	β -GAL ACTIVITY
pRS423.21-1	m4	3.3
pRS423.21-1	NaII	4.8
pACT2.DomNeg	m4	32.4
pACT2.DomNeg	NaII	34.1
pACT2	m4	0.2
pACT2	NaII	0.3

Activation levels of CYC1-LacZ reporter driven by high and low copy GAL4 Activation domain/REST DNA binding domain fusion proteins.

FIGURE 9 Western analysis of REST DNA Binding Domain expressed at high and low levels in yeast.

A) Two REST DNA Binding Domain fragments were expressed at different levels from high copy (2u) plasmids. pACT2.DomNeg expresses the gal4 activation domain fused to the RE1 DNA binding domain derived from mouse REST/NRSF from the moderate strength ADH1 promoter. pRS423.21-1 expresses the gal4 activation domain fused to a region of mouse REST/NRSF encompassing the DNA binding domain from a weak cryptic promoter in the vicinity of the pRS423 polylinker.

Lane1 contains 10 μ g extract from yeast bearing pRS423.

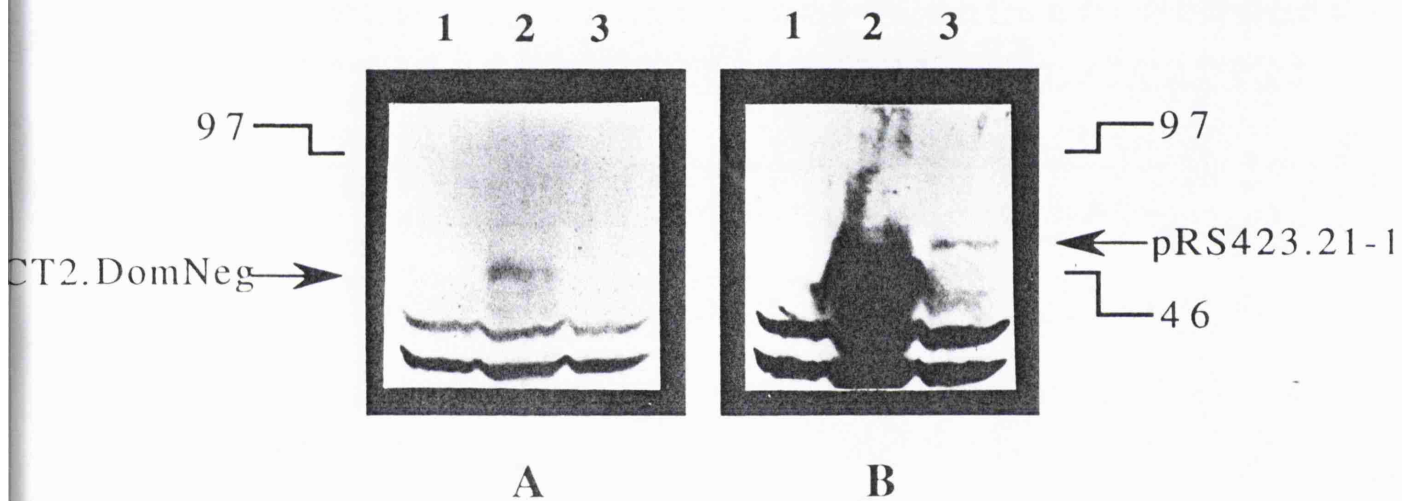
Lane2 contains 10 μ g extract from yeast bearing pACT2.DomNeg.

Lane3 contains 10 μ g extract from yeast bearing pRS423.21-1.

Western was probed with anti-gal4 activation domain antibody (Clontech) and exposed to X-ray film for 30 seconds.

B) Exactly the same as above but shows a longer exposure autoradiograph (20 minutes) to show faint band due to low expression from pRS423.21-1.

Molecular weights are shown on side of autoradiograph.



The experiments performed thus far suggest that such differential expression of both RE1 containing genes is unlikely to be a manifestation of the differential affinities of the two sites for REST, rather a function of other elements and factors working on the core promoters of the respective genes. It is however possible that even though REST by itself shows no site preference for the *NaII* RE1 and *m4* RE1 sequences, its affinity for one or the other site may be modulated either in a positive or negative manner by other auxiliary factors. This mode of transcription factor/element affinity modulation has been characterised for the drosophila homeodomain protein *fushi tarazu* (*ftz*) regulatory protein *ftz-F1* (Yu *et al.*,1997). The gene *ftz-F1* encodes a nuclear hormone receptor which acts to enhance the binding of *ftz* to its cognate element allowing *ftz* to bind weak sites which poorly bind at low concentrations. It is thought that co-operative interactions between homeo-domain proteins and cofactors of different classes may serve as a general mechanism to increase homeodomain protein specificity. It is possible that such affinity modulators could be at work with a host of other transcription factors including REST. This would allow subtle difference in the affinities of the RE1 element to be utilised to maximise the expression repertoire of RE1 containing genes without having to invoke the use of other transcription factors.

APPROACHES TO MODIFY THE 1-HYBRID SCREEN

A number of variations to the basic 1-hybrid protocol were attempted in order to minimise the number of false positives that arise due to events other than true 1-hybrid interactions. The initial screening regime was to plate the library transformed SFY526 yeast strain onto plates lacking tryptophan to maintain selection for the reporter plasmid and lacking histidine to select for reporter gene

expression. Colonies would then be assayed for the presence of library by plating onto plates lacking leucine - the assumption being that histidine autotrophs lacking a library plasmid cannot be harbouring a true 1-hybrid interaction and those that do have a library plasmid may be harbouring a true interaction. These would then be analysed further. However, 2×10^6 yeast transformants on plates lacking tryptophan and histidine resulted in ca 500 colonies. 100 of these were assayed for library plasmid. None passed this test.

Histidine autotrophy in this strain cannot arise due to gene conversion events between the reporter plasmid borne HIS3 coding sequence and mutant chromosomal locus because SFY526 harbours the his3 Δ 200 non-reverting mutation. The mutation is a complete knockout of 5' and 3' flanking sequence and all the coding sequence making gene conversion impossible. Histidine autotrophy thus has to arise through mutations that up-regulate the GAL1 promoter. Such mutations have been described that allow upregulation of promoters in the absence of their cognate activators e.g. the TBP mutant mot1 and the chromatin remodelling STP mutants (see 'Introduction'). If we assume that a particular locus undergoes a mutation at a rate of 10^{-7} /cell/generation then plating a 500ml culture at density= 2×10^7 cells/ml will generate ca 1000 histidine autotrophs in a library independent manner. This number tallies well with the observed figure of 500. Initial screens were performed with a transformation efficiency of ca 5×10^3 /ug library DNA/500ml OD₆₀₀=2 culture. Thus 2×10^6 transformants required 500mls and 500ug DNA. So initially ca 1 in 5×10^3 cells were transformed. Recently, efficiencies in our hands have increased 100 fold using more tightly controlled preparations of carrier DNA and transforming strains at cell densities optimal for each strain. Thus now

approximately 1 in 50-100 cells are transformed. At this efficiency, the above screening regime may be viable since 100 fold fewer cells need be plated to obtain the same number of transformants and since library independent histidine autotrophy is a function of cell number, approximately 100 fold fewer mutants will need to be screened.

In order to lower the mutant background, I attempted to perform the library screen in a diploid strain. Since the bulk of relevant mutations are recessive, they would not be seen in a diploid cell. However, mating of SFY526 with Y190 yielded a diploid strain which was very refractory to high efficiency transformation yielding approximately $10^3/\mu\text{g}$ DNA. Screening 2×10^6 transformants would thus require a prohibitive 2mg of library DNA! However, if a diploid strain with a high transformation efficiency could be generated, this approach would be very attractive.

The product of the GAL80 gene encodes a repressor that binds and masks the gal4p activation domain in the presence of galactose (see 'Introduction'). Also, a true 1-hybrid interaction can be defined as an interaction that recruits the GAL4 AD to the reporter promoter. Therefore, true 1-hybrid induced histidine autotrophy should be susceptible to gal80p mediated repression in the absence of galactose and thus fail to grow in the absence of histidine. I attempted to exploit this gal80p susceptibility to rapidly screen colonies that arise in 1-hybrid screens. I performed a 1-hybrid screen in the GAL80⁺ strain FM242 and picked 10 colonies out of 50 that grew on galactose/raffinose plates lacking tryptophan, leucine and histidine supplemented with 5mM 3-AT. These 10 candidates were sequenced and assayed for a true 1-Hybrid interaction by retransformation into the above strain and also SFY526. All 10 failed to show an interaction.

The original colonies were then replica plated onto triple drop-out plates containing raffinose supplemented with increasing 3AT concentrations. Raffinose prevents mig1p mediated repression (due to the absence of glucose) but induces gal80p mediated repression (absence of galactose). No differences were seen between the growth abilities of the 10 non 1-hybrid candidates and pACT2DN. The fact that pACT2DN grew suggests that gal80 mediated repression was not functioning.

FM242 containing a reporter plasmid with consensus gal4p binding site (cUAS) showed much reduced growth on raffinose plates compared to plates containing galactose and raffinose. This suggests that gal80p mediated repression is functional when acting on endogenous gal4p. However, over-expressing gal4p using the plasmid pCL1 (expresses full length gal4p from the wild type GAL4 promoter) caused a marked reduction in gal80p mediated repression in FM242. It is likely therefore that no gal80p mediated repression was observed with pACT2DN because gal80p was quenched by the over-expressed gal4 activation domains in the fusion protein driven by the moderate ADH promoter. To test this hypothesis I assessed the extent of gal80p mediated repression in FM242 expressing just the gal4 activation domain from the plasmid pGAD10. As expected, no difference in growth and thus no difference in gal80p mediated repression was seen between cells grown on galactose/raffinose and those grown on just raffinose. The failure of this approach is thus attributable to the low levels of endogenous gal80p in the cell. Driving GAL80 expression from a stronger promoter should overcome this difficulty. This would allow the very rapid screening of 1-hybrid candidates by performing the initial screen in a Gal80⁺ strain in the presence of galactose and raffinose and

then replica plating onto raffinose plates. Those colonies that failed to grow in the absence of galactose would be candidates for true 1-hybrid interactions.

The potential draw back of the above approach is the following: a number of DNA binding proteins have been isolated using the 1-hybrid assay where the DNA binding domain is not in frame with the gal4 activation domain. The protein is either encoded on the sense strand but out of frame or on the antisense strand e.g. MIG2 (Lutfiyya and Johnston,1996). Transcription and translation thus occurs in a 'cryptic' fashion to produce the protein. Activation occurs by uncharacterised interactions with the core complex in such cases but importantly does not require the gal4 activation domain. These candidates would thus be missed in the GAL80 mediated repression screen.

The final modification attempted to the 1-hybrid screen was based on counter-selection against background events. Because, gene conversion at the HIS3 locus is not possible in the strains used, the only route to histidine autotrophy other than via a true 1-hybrid interaction is to undergo an activating mutation to upregulate the unactivated GAL1 promoter driving HIS3 expression. I reasoned that such mutations should also up-regulate the chromosomal GAL1 promoter driving the GAL1 gene and possibly other TATA containing promoters. This is because the pre-initiation complex recruited to the plasmid borne GAL1 promoter should be the same as the chromosomal GAL1 promoter since both have identical sequence.

2-deoxy-galactose is lethal to cells expressing GAL1 due to the toxic accumulation of 2d-gal-6-phosphate. I reasoned that any cells harbouring mutations to upregulate GAL1-HIS3 should also then

upregulate GAL1-GAL1. If this is so then the presence of 2d-gal would kill these mutants. True 1-hybrid interactions however would only up regulate GAL1-HIS3 because only the reporter plasmid has the appropriate UAS and GAL1-GAL would be silent therefore these cells would not be 2d-gal sensitive. I plated 10 colonies isolated from a 1-hybrid screen SFY526 known not to harbour 1-hybrid interactions onto triple drop out plates containing 0.1% 2d-gal. SFY526 bearing pACT2DN was also plated onto these plates. All colonies grew irrespective of whether they harboured a true interaction or not. It is not clear why those colonies with, presumably upregulated GAL1 promoters, grew in the presence of 2d gal. One possibility is that the extent of activatorless GAL1 activity, though enough to support histidine autotrophy, is not enough to make cells 2d-gal sensitive. Plating on higher concentrations of 2d-gal could possibly allow counter-selection of false positives but the extreme cost of this reagent would make such a protocol prohibitive.

In summary, I have screened three libraries for RE1 binding proteins using the yeast 1-hybrid protocol. Further, a number of modifications have been attempted to improve the screen and reduce the background events that hinder rapid isolation of true 1-hybrid candidates. The avenues explored should be useful as a basis to perfect the screen and reduce background to a minimum.

CHAPTER 5.

ANALYSIS OF REST REPRESSION OF THE GAL1 PROMOTER IN YEAST

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ANALYSIS OF REST REPRESSION OF GAL1 IN YEAST

INTRODUCTION.

Yeast have proved to be a very important tool in establishing many paradigms in mechanisms of gene regulation. Many components and mechanisms of gene activation and repression found in yeast also act in higher eukaryotes. For example, the basal transcription factors e.g. TBP and TAFs belonging to the TFIID complex are very similar across yeast and mammals. Further many other mammalian transcription factors have yeast homologues e.g. hDR1 and hDRAP1 are human homologues of YDR1 and BUR6 (Kim *et al.*,1997). This conservation of mechanism has been used to isolate higher eukaryote transcription factors using screening methodologies in yeast; the *drosophila* gene HMG-14 which modulates the activity of the protein Dorsal was found by screening *drosophila* cDNA libraries in yeast (Lehming *et al.*,1994).

Like many promoters found in mammalian cells, the promoter driving expression of the yeast GAL1 gene is under the control of both positive and negative regulatory factors. Glucose is the preferred carbon source for *S.cerevisiae* and as such, the galactose utilisation pathway is tightly glucose repressed along with other pathways such as gluconeogenesis (Johnston *et al.*,1994; Johnston,1987; Trumbly,1992). The GAL1 promoter is activated in the presence of galactose by the acidic activator gal4p which binds to the UASG sites upstream of the GAL1 TATA box or Upstream Essential Sequence (UES) (Johnston *et al.*,1987). The gal4p is present at very low levels in the cell but those

levels themselves are regulated by the absence or presence of glucose. In the absence of galactose, the levels of gal4p are down regulated resulting in decreased levels of GAL1 transcript. Further, any remaining gal4p bound upstream of the GAL1 promoter are 'capped' by gal80p preventing residual activation of the GAL1 gene in the absence of galactose (see 'Introduction'). The GAL1 gene is further strongly repressed by the presence of glucose. This is achieved through the action of the protein encoded by the MIG1 gene. In the presence of glucose mig1p, a zinc finger repressor, binds the Upstream Regulatory Sequence (URSG) in the GAL1 promoter to shut down transcription.

Binding of mig1p to URSG is SNF1 dependent suggesting that mig1p action is regulated by phosphorylation. Further, repression by the MIG1 gene product is dependent on SSN6 and TUP1. Promoters containing functional URS sequences e.g. SUC2 and MAL1, can be repressed fully by recruiting the SSN6/TUP1 complex to the promoter via fusion to a lexA DNA binding domain suggesting that SSN6/TUP1 acts as a co-repressor complex.

Rat m4 transcription is driven by a constitutive TATA-less/CAAT-less promoter which is at least partly repressed in non-m4 expressing tissue by the Zinc finger repressor REST/NRSF (see Chapter3 and Chapter 4). In order to dissect the molecular mechanism by which REST mediates repression occurs, I have attempted to use the conserved aspects of gene regulation between yeast and mammals. I have expressed REST in yeast containing an RE1 site upstream of GAL1-HIS3 and show that REST is able to repress transcription of the gal4p activated GAL1 promoter. Further molecular analysis is described to attempt an understanding of the mechanisms of REST mediated repression in yeast.

RESULTS AND DISCUSSION.

Upon finding that repression of the m4 promoter was mediated by REST, we sought to investigate further the molecular mechanisms that underlie REST action. A molecular description ultimately requires a picture of the protein-protein and protein-DNA interactions within a REST repressed (and unrepressed!) promoter.

The study of repression of transcription in yeast has been greatly helped by the ability to use genetic approaches and library screening protocols in this organism - feats impossible when studying repression in cell lines or mice. I thus attempted to set up a system in yeast which would allow me to characterise the modes of REST action at a promoter.

To assess the ability of REST to repress a yeast promoter, I expressed HZ4 (a truncated REST clone containing the N-terminal repression domain, Zn fingers and Lysine rich region) in yeast. This molecule was able to repress a gal4p activated GAL1-HIS3 reporter construct bearing 3 RE1 sites in between the UAS and TATA element. The extent of repression was very strong as judged by the inability of the yeast to grow in the absence of 3-AT (figure 10b) The DNA binding domain of hREST showed no repression. Interestingly, expression of mREST in yeast failed to show any repression despite Western analysis showing that both HZ4 and mREST were expressed (data not shown). One possibility is that there is a species to species difference in the function of REST. However, analysis of the N-terminal repression domains and Zn finger region sequence shows 91% identity over the N-terminal 143 residues (see figure 11). Another difference between the

FIGURE 10. Repression of GAL1-HIS3 by REST in yeast.

Figure A (top), schematic diagram of REST and partial REST constructs used in GAL1 repression assays.

Figure B (bottom), growth assay on galactose/raffinose plates lacking histidine of yeast expressing HZ4, DomNeg, HZ4D1-138 or containing empty expression vector on 1.5mM 3-AT (left) or 10mM 3-AT(right). Yeast colonies harbouring the indicated pLeonov expression plasmid and pBM2389(RE1)₃UAS reporter plasmid were streaked onto the appropriate selective plates and growth was assessed after 3 days at 30°C.

Inset, reporter plasmid {pBM2389(RE1)₃UAS}used for repression assay.

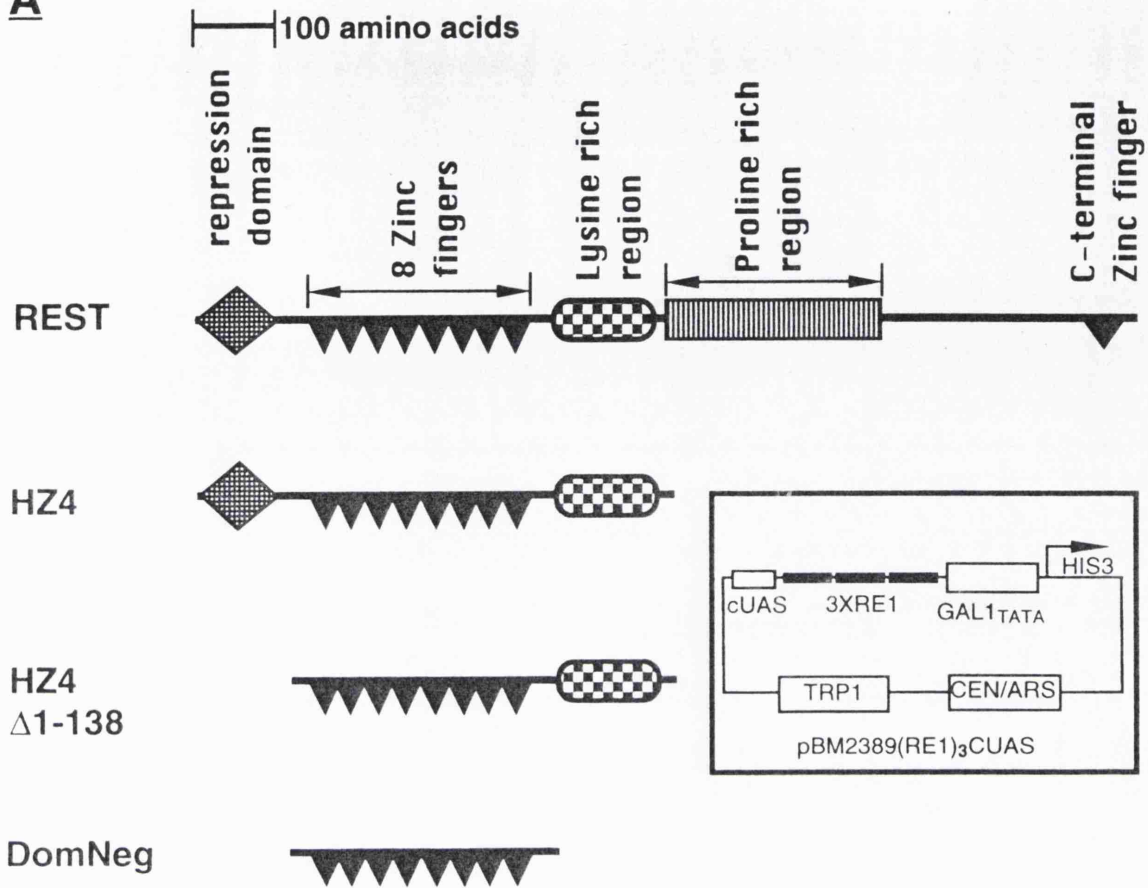
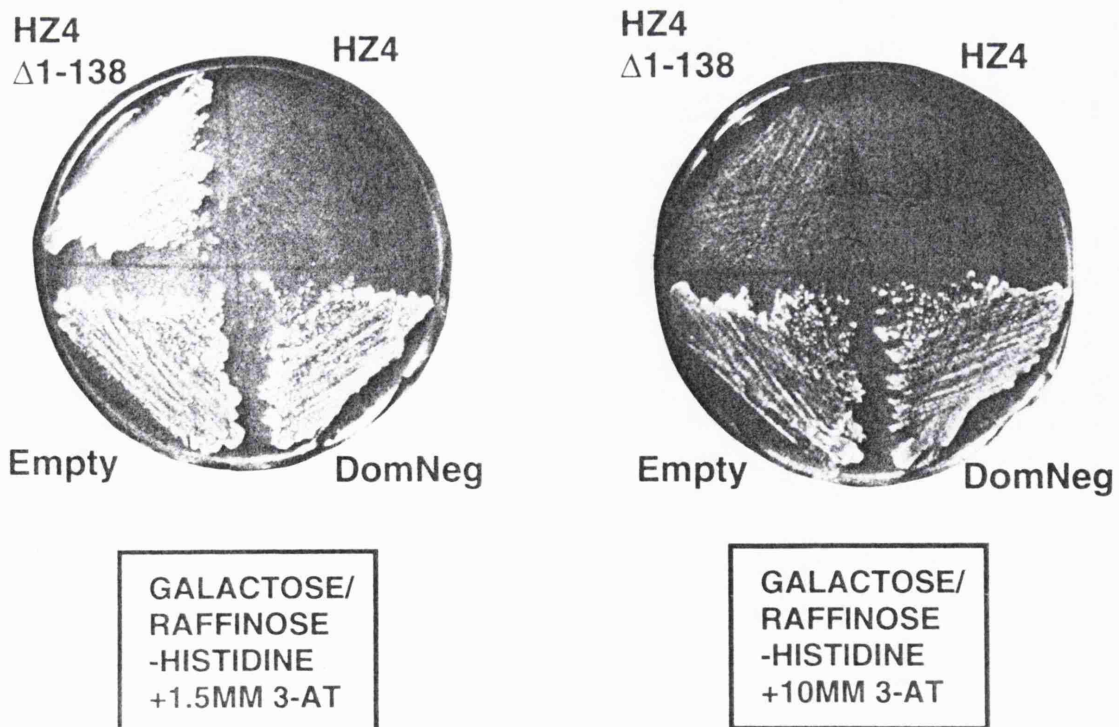
A**B**

FIGURE 11 Human and mouse REST are highly conserved within the N-terminal repression domain, DNA binding domain and lysine rich region.

Human and mouse REST sequence were compared using the GCG PileUp program (Staden software accessed via hgmp). For boundary definitions see figure 7 and figure 10a.

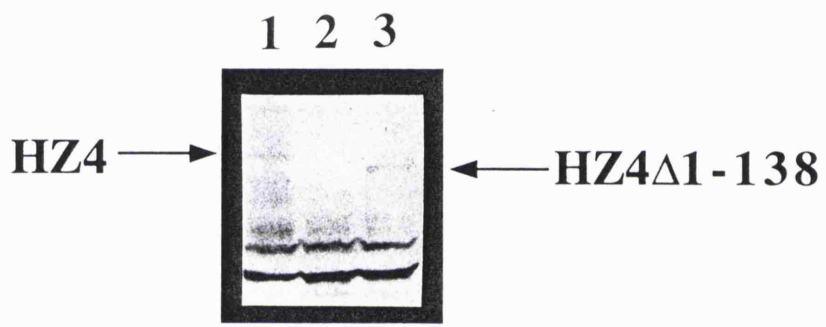
2 constructs is the size. HZ4 is less than 65KD while mREST is 116KD. Folding of the larger protein may be problematic and thus even though Western analysis with antibody against the sequential myc epitope shows expression of all constructs, mREST may not be functional.

Having shown that HZ4 was able to repress in yeast, I wanted to confirm that the mechanism of REST mediated repression was the same as in mammals. The repression domains of REST functional in the mammalian cell line PC12 had already been described by Tapia-Ramirez and co-workers as the N-terminal 77 amino acids and also the C-terminal Zn finger (Tapia-Ramirez *et al.*,1997). Both of these domains could function independently. The HZ4 expressing construct lacked the C-terminal Zn finger and so if the mechanisms of repression are conserved between mouse and yeast then GAL1 is repressed by the N-terminal 77 amino acids. To confirm this I removed the N-terminal 138 amino acids and replaced the translational start site with a myc tag to generate HZ4 Δ 1-138. Western analysis showed the HZ4 Δ 1-138 construct was expressed at similar levels to full length HZ4 as is seen in figure 12. When expressed in the reporter strain, strong growth was seen in the presence of 5-10mM 3-AT. At higher concentrations, stunted growth was seen suggesting residual repression activity. It is possible that this may be due to steric hindrance of gal4p binding to the UASG placed immediately upstream of the RE1 sites. However, I feel that this is unlikely since the DomNeg construct binds RE1 sites and still allows access of gal4p to the UAS as judged by potent growth at greater than 50mM 3-AT. The much reduced repression activity of the N-terminal deleted construct is consistent with there being mechanistic conservation of REST mediated repression between mouse and yeast. Interestingly, the work of Tapia-Ramirez *et al* shows that in PC12 cells

FIGURE 12. Western analysis of HZ4 and HZ4 Δ 1-138 expression in the yeast strain FM242.

Western blot of FM242 expressing HZ4, HZ4 Δ 1-138 or containing empty expression vector probed with anti-myc (9E10) antibody (Santa cruz) primary antibody and HRP conjugated anti-mouse IgG as secondary. Bands were visualised by ECL staining.

- Lane 1) 10 μ g extract from yeast bearing pLeonov.HZ4
- Lane 2) 10 μ g extract from yeast bearing pLeonov(HX)
- Lane 3) 10 μ g extract from yeast bearing pLeonov.HZ4 Δ 1-138.



with the NaII promoter as reporter, deletion of the N-terminal 73 amino acids of full length REST results in ca 60% loss of repression. This partial loss of repression is consistent with my findings in that removal of this region gives partial but not total loss of repression.

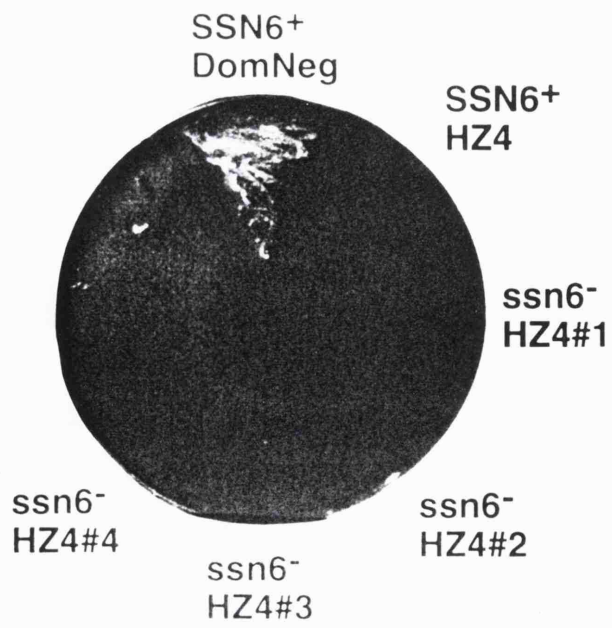
The GAL1 promoter is tightly regulated according to the carbon source availability of the yeast. In the presence of glucose, the protein mig1p binds the upstream regulatory sequence URSG. Mig1p represses the GAL1 promoter by recruiting the SSN6/TUP1 corepressor complex. In an SSN6 or TUP1 delete background there is one hundred percent loss of MIG1 mediated repression at the GAL1 promoter. Synthetic recruitment of SSN6 to GAL1 via fusion of a lexA DNA binding domain to SSN6 in a TUP1 expressing background allows efficient repression but not in a TUP1 delete background. However, recruitment of TUP1 to GAL1 via lexA allows repression in an SSN6 independent manner. These experiments suggest that mig1p binds SSN6 directly, and that the tethered TUP1 interacts with the repression machinery to mediate silencing.

In wild type yeast, repression of GAL1 is thus achieved by mig1p binding its cognate DNA element and so recruiting the SSN6/TUP1 repressor complex. I postulated that maybe REST mediated repression of GAL1 might work via recruitment of the same complex. Therefore I performed the repression assay in FM1433, an SSN6 delete strain. Potent REST mediated repression was observed as judged by lack of growth of HZ4 expressing yeast even on 1.5mM 3-AT (figure 13). This suggests that REST repression in yeast is not mediated by SSN6. Further, it is well documented that loss of a protein from a heterodimer complex often results in the degradation of the partner of that protein. It is therefore likely that REST repression is not mediated by TUP1

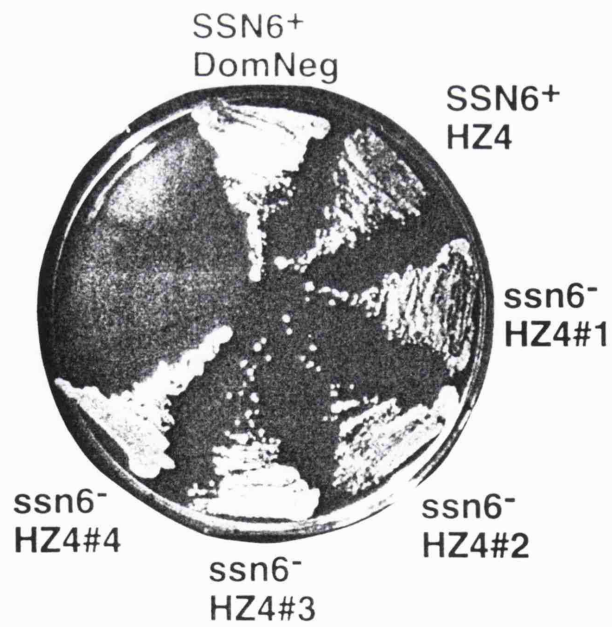
FIGURE 13. REST mediated repression in yeast is SSN6 independent.

Top: growth assays of SSN6⁺ and ssn6⁻ yeast expressing HZ4 or DomNeg on 2% galactose/2 %raffinose plates lacking histidine supplemented with 1.5mM 3-AT. Clockwise: DomNeg expressed in SSN6⁺ strain, HZ4 expressed in SSN6⁺ strain, streaks of four ssn6⁻ colonies expressing HZ4.

Bottom: Exactly the same as above streaked onto 2% galactose/2% raffinose plates supplemented with histidine with no added 3-AT.



GALACTOSE/
RAFFINOSE
-HISTIDINE
+1.5MM 3-AT



GALACTOSE/
RAFFINOSE
+HISTIDINE

either. Proof of this was difficult to substantiate due to the very sick nature of the TUP1 delete strain. TUP1 has not been implicated as far as I am aware in any SSN6 independent repression mechanisms. Therefore it is likely that a lack of dependence of REST repression in yeast on SSN6 probably extends to a lack of requirement for the SSN6/TUP1 co-repressor complex.

Repression of many other yeast genes e.g. HO, INO1, SPO11, SPO13, TRK2 and IME2 is mediated by the SIN3/RPD3 corepressor complex (see 'Introduction'). LexA fusions of SIN3 allow efficient repression of reporter constructs bearing a *lexA* operator in an RPD3 background but not in an *rpd3⁻* background (Wang and Stillman,1993). However, *lexA* fusions of RPD3 allows repression even in a *sin3⁻* delete background. Both *in vivo* and *in vitro* interaction assays show that the mammalian protein MAD interacts directly with *sin3p*. This suggests that *sin3p* binds to DNA bound transcription factors and in doing so recruits the silencing activities associated with RPD3, a histone de-acetylase. I assessed the possibility that REST may repress by recruiting the SIN3 corepressor complex. I generated a strain containing a URA3 disruption of SIN3 and performed the repression assay with HZ4. Complete abolition of repression was observed in the *sin3* background as judged by the ability to grow at the highest (25mM) 3-AT concentration tested in the presence of galactose and raffinose as carbon source. Full activity of the reporter GAL1:HIS3 construct was seen in the presence of both HZ4 and the DomNeg construct (figure 14a). This suggests that repression by REST in yeast is mediated via a pathway containing *ySIN3*.

In order to rule out the possibility that disruption of SIN3 in this strain resulted in a non-specific de-regulation of the GAL1 promoter I

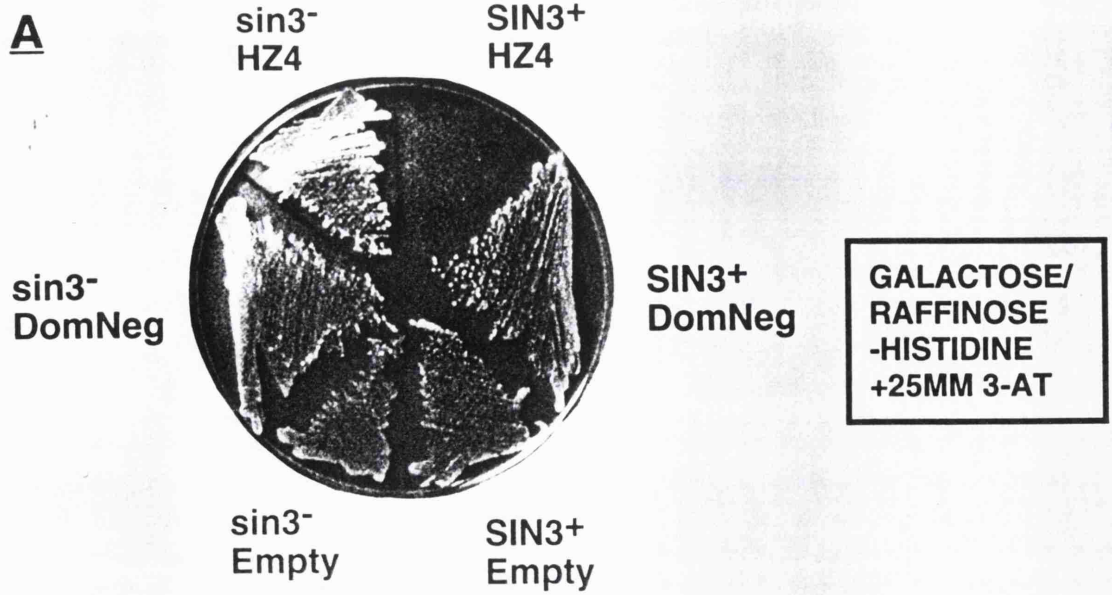
FIGURE 14. SIN3 disruption in yeast abolishes REST mediated repression but leaves glucose repression intact.

Figure A (top), growth assays of SIN3⁺ and sin3⁻ yeast expressing HZ4, DomNeg or empty expression vector on galactose/raffinose plates lacking histidine supplemented with 25mM 3-AT .

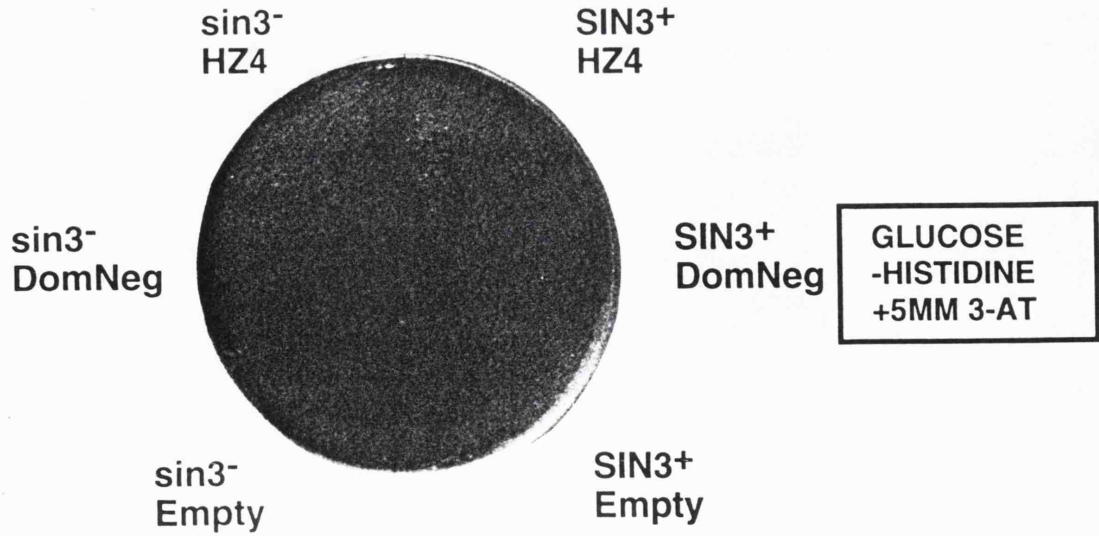
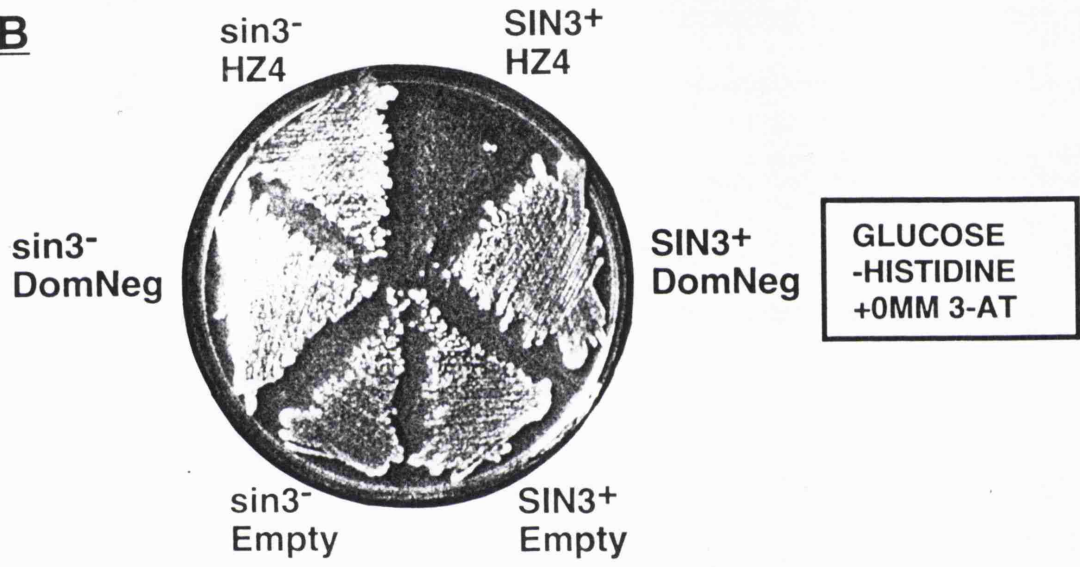
Figure B (bottom), growth assay of SIN3⁺ and sin3⁻ yeast expressing HZ4, DomNeg or empty expression plasmid on glucose plates lacking histidine supplemented with 0mM 3-AT (middle) or 5mM 3-AT (bottom).

Growth was assessed after 3 days at 30°C.

A



B



assessed the integrity of the glucose repression mechanism on the GAL1 promoter. In the presence of glucose at 0mM 3-AT in both wild type and *sin3⁻* strains basal levels of HIS3 expression were observed in the presence of the DomNeg construct and empty expression vector. However, while as wild type yeast showed complete absence of basal activity in the presence of HZ4, *sin3⁻* cells showed basal expression (figure 14b). This suggests that the ability of HZ4 to abolish basal activity of the GAL1 promoter as well as activated expression is SIN3 dependent. At 5mM 3-AT in the presence of glucose, no growth was seen for either strain with any construct suggesting that glucose repression was still intact in the *sin3⁻* strain.

Whether, *ySIN3* directly interacts with REST has yet to be established. Tagging the endogenous *ySIN3* gene with a HA epitope by recombination and performing a 'GST-pull down' experiment with a GST-REST fusion would clarify this issue.

Mammalian Mad protein as a heterodimer with Max represses promoters via the E-box motif by recruiting a SIN3/N-CoR/RPD3 complex. In yeast, recombinant Mad couples to *ySIN3* via its SIN3 Interaction Domain (SID) (see 'Introduction'). It is tempting to think that REST acts in an analogous manner and so interacts directly with *ySIN3*. Sequence analysis fails to show a SID in HZ4, however UME6 interacts with *ySIN3* without possessing a SID, therefore, absence of SID in REST does not preclude a REST/SIN3 interaction.

The mammalian relevance of these findings in yeast have to be assessed. One approach will be to see if *mSIN3A* and/or *mSIN3B* interact with REST in yeast. *mSIN3A/B* fails to recapitulate MAD repression in a *ysin3⁻* background in yeast (Kasten *et al.*,1996). It is

known that MAD interacts with mSIN3 via PAH2 both in mammalian systems and in yeast, therefore, failure of mSIN3 to complement ysin3 is likely to reflect a failure of mSIN3 to communicate with the downstream repression machinery. This precludes an analysis of mSIN3A/B-REST interactions in yeast by complementation of ysin3. However, it may be possible to generate a GAL4AD-mSIN3A/B fusion and express this in a ysin3⁻ background to see if HZ4 is converted to an activator in the presence of mSIN3A/GAL4AD and/or mSIN3B/GAL4AD. These experiments will show whether REST is able to interact with mSIN3A or B or indeed both.

CHAPTER 6.

DISCUSSION

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DISCUSSION

In this study I have shown that the m4 muscarinic receptor gene is under the control of a constitutively active core promoter and that cell specific expression is achieved through selective repression in m4 non-expressing cells. I show that repression of m4 expression is mediated by REST as well as by as yet uncharacterised transcription factors.

I have also demonstrated that REST can repress the GAL1 promoter in yeast. Further, I establish that the ySIN3 gene is essential for REST mediated repression in yeast and that the global repressor complex SSN6/TUP1 is dispensible for repression. Arguments are presented to suggest that REST may work via both acetylation/de-acetylation dependent and independent mechanisms and that REST itself may be modified by acetylation.

Transient Transfection Analysis.

In the m4 non-expressing non-neuronal cell lines CHO and 3T3 and m4 non-expressing neuronal cell line NBOK1, repression is mediated at least in part by an RE1/NRSE element situated at position -574/-550. It is also apparent that at least in the fibroblast cell line 3T3, the m4 gene is under the control of two promoters (giving rise to two transcription start sites, tsp-u (upstream) and tsp-d (downstream)). Further, tsp-u is under the control of the RE1 element, presumably via REST binding, and tsp-d repression is not mediated via this element.

Therefore, silencing of the m4 gene in 3T3 cells requires another repressor element(s) which is either not present in the largest construct used or is unable to function in transient transfection assays.

Examples exist of other genes with two promoters both in yeast and mammals. The yeast HIS3 and GAL80 genes both possess 2 promoters(Chen and Struhl,1985),(Sakurai *et al.*,1994). In the case of HIS3, the +13 site is driven by a TATA containing promoter which is activated by the as yet unidentified activator of HIS3. The +1 site is driven by a constitutive TATAless promoter whose expression level is unaffected by the absence or presence of HIS3 activator. So, like the m4 gene, in 3T3 cells, HIS3 is under the control of two differentially regulated promoters.

It is not immediately obvious why a gene should be regulated by two distinct promoters if transcription from either promoter gives rise to the same protein, as in the case of the m4 gene. However, a cell may have many genes sharing common elements, such as RE1. For example, if all these genes except for m4 are to be silenced in a given cell type except for m4, for example, then a parsimonious cellular strategy might be to express REST in order to silence all REST repressible promoters but to endow m4 with a second 'REST immune' promoter to allow continued expression. This may be more economical than placing all other genes under the control of individual elements.

Whether this is a reason for having two promoters with disparate responses to REST is arguable. In the adult nervous system, m4 is expressed mainly in the telencephalon and autonomic ganglia and is silent everywhere else. It is unlikely that REST mediates this repression in the nervous system because neuronal REST levels are only found at

high levels in neuroepithelia during embryogenesis. However, the argument may still apply if we assume that REST responsiveness of the two promoters is merely indicative of other differences between the two recruited complexes. In other words, the different responses to REST underlie a fundamental regulatory difference between the promoters which extends to other as yet uncharacterised factors and that these factors may decide whether m4 is expressed in one adult neuron over another.

The fact that REST is not expressed in differentiated neurones and m4 is only found in a subset of telencephalic neurones and autonomic neurones, suggests that there are cells which do not express REST and do not express m4. Therefore the m4 gene must be under the control of other elements and factors. In the m4 non-expressing neuronal cell line Neuro-2a, repression must be mediated by an element that is either not in the largest construct used in the analysis or is present but unable to operate in a transient transfection assay. The RE1 element is unable to mediate repression in this cell line. I have shown that this lack of RE1 mediated repression is not due to limiting amounts of REST in Neuro-2a cells because co-transfection of a REST expressing plasmid fails to recapitulate repression (see figure 4). This suggests that i) repression is mediated by another element (with the caveats mentioned above) ii) that the core complex recruited to the m4 promoter in this cell line is functionally distinct from that recruited in the other cell lines used in the study in that it is refractory to REST mediated repression iii) REST is modified in some manner in this cell line to make it inactive. Also, the REST DNA binding domain has a similar affinity for both the NaII RE1 element and the m4 RE1 element yet the NaII gene is expressed in NEURO-2A cells while as the m4 gene is silent. If this differential

expression of two RE1 containing genes is due to the low levels of REST binding the elements differently, then one would expect the m4 RE1 element to have a higher affinity for REST than the NaII element. 1-hybrid analysis of REST DNA binding domain for the two elements shows this not to be the case.

Because transiently transfected plasmids are not packaged into ordered chromatin (Archer *et al.*,1992), certain aspects of endogenous gene regulation may fail to be recapitulated in the above experiments. Hence, although it is likely that the core promoter complexes recruited to the transient templates are the same as those recruited to the chromosomal gene, they may nevertheless be subject to different regulatory factors. As such, if REST can mediate repression via chromatin remodelling and also, by direct repression of core complexes (as is discussed below) then only the latter arm of REST repression will be observed in a transient transfection assay. So if in 3T3 cells, *tsp-u* is repressed via REST directly inactivating the recruited core complex and *tsp-d* is silenced by chromatin remodelling as is the complex recruited in Neuro-2A cells, then in transient transfection assays, REST would be seen to repress *tsp-u* in 3T3 cells but not *tsp-d* or the Neuro-2A complex.

Determining whether all aspects of REST mediated repression are recapitulated in transient transfection assays is important because if they are, then other repressor elements must be invoked which silence *tsp-d* expression and expression in NEURO-2A cells. If however, it is found that REST cannot act on transient templates in the same manner as chromosomal templates, then other repressor elements may not be required to explain silencing of endogenous promoters in these cell lines. Obviously, other repressor elements are still required in silencing

m4 expression in those tissues *in vivo* that do not express REST and do not express m4.

Analysis of endogenous m4 transcripts in 3T3 cells in the presence and absence of REST would potentially allow discrimination between the above models. Reduction of REST levels in 3T3 might be achieved by transfecting cells with REST antisense phosphothiolated oligonucleotides to REST transcript. RNase protection analysis with a probe spanning from downstream of tsp-d to upstream of tsp-u would distinguish between both or just one of the promoters being up-regulated. This approach would need a high transfection efficiency to give detectable signal. The potential draw back would be that general reduction in REST levels may produce pleiotropic effects due to upregulation of other REST regulated genes, some of which may themselves be as yet uncharacterised regulators of m4 expression.

Mechanisms Of REST Repression: Development Of A Yeast Model.

Having established that REST is at least partially responsible for repression of the m4 gene, I initiated a study into the molecular mechanism(s) that REST employs to mediate repression. I expressed a human REST fragment, HZ4 (residues 1-599) which had been shown to repress the SCG10 gene in transient transfection assays (Schoenherr and Anderson,1995), in yeast bearing a GAL1-HIS3 reporter construct. This fragment was able to silence both the activated GAL1 promoter (in the presence of galactose) and basal GAL1 promoter (in the presence of glucose) in an RE1 site dependent manner. The ability of REST to repress in yeast suggests that either the machinery REST uses to repress is conserved through evolution or that REST is able to work through

different mechanism in yeast and mammals to mediate repression. The significant but incomplete loss of repression upon removal of the N-terminal repression domain in yeast (see 'Chapter 5') is in accordance with the significant but not total loss of repression (60%) that removal of this domain from REEX7 (contains an extra 35 residues C-terminal to HZ4) causes in transient transfection experiments in mammalian cell lines using the NaII (see Tapia-Ramirez *et al*,1997). This is consistent with there being mechanistic conservation between yeast and mammals with respect to REST mediated repression.

It was the anticipation of mechanistic conservation that prompted this line of research. The ability to perform sophisticated genetic manipulations in yeast, for example gene knock-outs, homologous recombination and library screening in a routine and rapid manner is unprecedented in mammalian systems. As described in the 'Introduction', many facets of transcriptional regulation in higher eukaryotes can be reproduced in yeast. For example, DSP1 can convert Dorsal from a gene activator to a repressor. DSP1 was isolated from a drosophila cDNA library by counterselection for URA3 expression driven by a Dorsal activated GAL1 promoter bearing tandem VRE elements taken from 1.6Kb of Zen promoter sequence (Lehming *et al*,1994). Further, in the case of Dorsal, analysis of its ability to activate the GAL1 promoter showed that activation was dependent on the SWI/SNF complex (Winston and Carlson,1992). Thus, expression and genetic analysis of a heterologous transcription factor in yeast, coupled with the ability to perform rapid genetic modifications in this organism yielded an insight into the mechanism of Dorsal's ability to activate genes.

Having shown that there may be mechanistic conservation between yeast and mammals in terms of REST mediated repression I

attempted to identify yeast proteins involved in REST mediated repression. Using glucose repression of the GAL1 promoter as a model system I wanted to see whether, REST repression of GAL1 worked in an analogous manner to mig1p repression i.e. via recruitment of the SSN6/TUP co-repressor complex. For any gene to be a candidate for a mediator of REST repression, it should possess a co-repressor function and importantly have a mammalian orthologue. A human homologue of TUP1 had already been isolated by others and its loss implicated in the disorder known as DiGeorge Syndrome (Halford *et al.*,1993). A preliminary data base search has already shown that although no mammalian specific homologue of SSN6 has been characterised to date, many mammalian and human genes were identified in a database search which shared a glutamine repeat region e.g. SCA1 implicated in Machado-Joseph disease(Wanget *al.*,1997). Potent REST repressor activity was seen in FM1433, an *ssn6⁻* strain. This suggested that SSN6/TUP1 complex was not involved in mediating REST repression.

I next looked to see if REST repression in yeast was mediated by the SIN3 complex because, like TUP1, SIN3 fulfils the criterion of being a co-repressor and being conserved between yeast and mammals. Disruption of SIN3 in the yeast strain FM242 resulted in potent activity of the GAL1 promoter in the presence of HZ4. This strongly implicates *sin3p* as being essential in mediating REST repression in yeast. Direct coupling of REST to *sin3p* has yet to be demonstrated but by analogy to other SIN3 dependent repressors, there is a strong possibility that such coupling occurs. For example, mouse Mad protein fused to *lexA* DNA binding domain can silence the GAL1 promoter by coupling to the product of the yeast SIN3 gene. The fact that REST does not possess a recognisable Sin3 Interaction Domain (SID) does not rule out SIN3

coupling. The yeast gene UME6 encodes a repressor that works via SIN3 yet does not possess a SID (Kadosh and Struhl,1997).

The product of the SIN3 gene in yeast is associated with rpd3p, a de-acetylase (See 'Introduction' and 'Chapter 5') and is recruited to promoters via DNA bound transcriptional repressors. If it transpires that REST mediated repression in mammals is also SIN3 dependent then it suggests that REST may repress by recruiting de-acetylase activity to target promoters. Until very recently, acetylase and de-acetylase activity was thought to only target the N-terminal lysines of Histones H2A, H2B, H3 and H4 (Turner,1993; Brownell and Allis,1996; Wolffe and Pruss,1996; see Luger *et al*, 1996 for structural information) However, p53 has now been implicated as a substrate for acetylase activity opening up the possibility that there are other non-histone substrates for this type of modification (see later). Aside from telomere effects (reviewed in Pazin and Kadonaga, 1997), hypo-acetylated histones tend to be associated with quiescent chromatin while as hyper-acetylated histones tend to be associated with transcriptionally active chromatin.

In mammals, the use of SIN3 containing repressor complex recruitment to modulate promoter activity has been implicated for diverse families of transcriptional repressors ranging from the bHLH-Zip proteins of the Mad repressors through to the steroid dependent nuclear receptor family of transcriptional repressors. In both cases, conversion from activator to repressor involves the exchange of an acetylase complex for a SIN3/N-CoR complex (see 'Introduction'). In the case of the steroid receptors the SIN3/N-CoR complex has an associated de-acetylase activity, mRPD3 (HD1). For the bHLH repressors, the SIN3/N-CoR complex may contain mRPD3 depending on the isoform of mSIN3B in the complex: mSIN3B_{SF} has no associated mRPD3 while as mSIN3B_{LF}

does (Alland *et al.*,1997). If a SIN3 complex is required for repression by REST in mammalian systems, then it will represent a third class of repressor that converges on this repression pathway.

Potential Role Of REST/SIN3 In Neuronal Development.

Proliferating mammalian cells tend to express high levels of myc and low levels of Mad. Differentiation sees a reversal of this profile such that the increased levels of Mad antagonise Myc binding to Max and thus by recruitment of a SIN3 complex, cause repression of target promoters involved in proliferation (Ayer and Eisenman,1993; Hurlin *et al.*,1995). So it would seem that SIN3 dependent repression is involved in terminal differentiation. In contrast, REST levels are elevated in proliferating neuroepithelia and very low in adult differentiated neurons (Schoenherr and Anderson,1995). As such, it would seem that REST and thus SIN3 complex, is not involved in the maintenance of the adult neuronal phenotype. REST expressed during neuronal development may be required to minimise unwanted neuronal gene expression during embryogenesis. So, unlike SIN3 dependent repression of target genes through differentiation by the bHLH class of proteins, REST may invoke SIN3 dependent repression to silence genes whose expression may be detrimental during proliferation of neuronal precursors.

Upon neuronal differentiation, REST levels become almost undetectable within the nervous system. The implication is that other mechanisms come into play to repress neuronal genes in those neuronal cells where they are not required. Further, loss of REST in differentiated neurons could potentially lead to liberation of SIN3 complex for acquisition by other classes of repressor such as the bHLH

family to mediate repression of promoters associated with proliferation. Such a model which invokes limiting amounts of co-repressor which is shared among diverse classes of repressor is suggested by Alland *et al*,1997 and Heinzl *et al*,1997 as a means of integrating repression/activation signals between the bHLH and nuclear receptor families. In their model, they suggest that exposure of proliferating cells to retinoic acid would convert Retinoic Acid Receptors (RARs) from repressors to activators, turn on genes associated with differentiated cells and liberate mSIN3/N-CoR/mRPD3 (and take up CBP/p300/p-CAF/p-CIP/NCoA-1 complex). This pool of SIN3 containing complex would then be acquired by increasing levels of Mxi associated with differentiating cells to mediate repression of genes required for proliferation. If such a model transpires to be correct, then liberation of SIN3 from REST in adult neurons may allow integration between signals that mark the onset of adult neuronal phenotype establishment (a process that is REST independent and thus able to liberate SIN3 complex) and the two arms of terminal differentiation, induction of RAR responsive genes and repression of bHLH responsive genes. It will be very interesting to know how many other repressors converge on this pool of co-repressor.

Mechanisms Of SIN3 Dependent Repression.

If SIN3 is shown to be essential for REST mediated repression in mammals, then how can REST mediate repression in transient transfection assays? Transiently transfected templates do not appear to be packaged into ordered chromatin (Archer *et al*,1992) and yet in yeast, SIN3 is associated with RPD3 and possibly other histone de-acetylases. Presumably, without chromatin, transiently transfected plasmids cannot offer histones as substrate for acetylase/de-acetylase

activity. This opens up two possibilities i) REST is able to mediate repression by a mechanism *in addition* to recruitment of SIN3 complex associated de-acetylase activity, ii) acetylase/de-acetylase activity is able to act on substrates other than histones and that this new target's acetylation state can modulate gene activity.

There is evidence that REST may operate by more than one mechanism. Deletion of the N-terminal repression domain in HZ4 resulted in only partial loss of repression of GAL1-HIS3, consistent with a second repression domain remaining in the construct pLEONOV.HZ4 Δ 1-138. This data cannot easily be explained by a model which states that the N-terminal repression domain represses via SIN3. If this was the case, then loss of SIN3 would have the same effect as loss of the N-terminal repression domain. Therefore, the N-terminus represses via a SIN3 independent pathway. Tapia-Ramirez *et al* (Tapia-Ramirez *et al.*,1997) show that the N-terminal repression domain is able to function in transient transfection assays in mammalian cells. This suggests that the N-terminus might repress by interfering directly, or indirectly with the pre-initiation complex and that some other part of REST (in my case HZ4) represses via SIN3. However, I feel that 'N-terminal repression' is the minor form of the two, at least in yeast, because of failure to see growth on plates lacking histidine supplemented with as little as 10mM 3-AT.

Determining which isoform of mSIN3 REST acts through will give a clearer picture of exactly how repression occurs and why that particular mode prevails over the alternatives. Mad and nuclear receptors act via interaction with a complex composed of N-CoR and mSIN3B. Further, Alland *et al* (Alland *et al* .,1997) show that at least Mad and Mxi can interact with either mSIN3B_{SF} or mSIN3B_{LF}. Interaction with the former

precludes interaction with mRPD3. This raises the possibility that REST may also be able to talk to either mSIN3A or mSIN3B. If the target is mSIN3B then REST could potentially recruit either an mRPD3 containing or mRPD3 lacking, N-CoR/SIN3 complex. Potentially, this may allow the unification of seemingly disparate observations: REST acts via SIN3 in yeast but also is able to repress in transient transfection assays in mammalian cells. One can imagine a scenario where *in vitro* repression by REST/mSIN3_{LF}/N-CoR/mRPD3 and REST/mSIN3_{SF}/N-CoR is mediated solely by virtue of N-CoR interacting directly with the core promoter complex. So the combined actions of the N-terminal REST repression domain and N-CoR may act on *in-vitro* templates to mediate repression while as *in-vivo* and in yeast, both arms of REST repression i.e. core complex repression and chromatin remodelling by mRPD3 can be elicited.

This would be very analogous to SSN6/TUP1 mediated repression in yeast. The SSN6/TUP1 complex is able to repress target promoters four fold *in vitro* but over 100 fold *in vivo* (Herschbach *et al.*,1994; Redd *et al.*,1997). This suggests that SSN6/TUP1 represses by multiple mechanisms to achieve full repression. One appealing aspect of this model of REST repression is that it allows the use of SIN3 isoforms in a way that does not involve chromatin changes which is normally associated with permanent differentiation. This would accommodate the notion that REST is present in neuroepithelia to minimise prodigious/detrimental expression of neuronal specific genes that are not required during embryogenesis (see above). This repression if mediated by mSIN3B_{SF} could be affected without resorting to chromatin remodelling, a process which tends to be associated with permanent

differentiation, but rather through the N-terminal repression domain and N-CoR acting on the pre-initiation complex of target promoters.

A further aspect of this model would be that if mSIN3B_{SF} is recruited by REST in neuroepithelia, then it becomes possible to endow certain RE1 containing promoters with core complexes that are either REST repressible or refractory to REST repression. This would allow a cell to express one RE1 containing gene yet silence another in the presence of REST. Such a situation would be hard to envisage if REST solely worked by imposing quiescent chromatin.

What of non-neuronal tissue? REST is found in adult non-neuronal tissue presumably to silence expression of neuron specific genes. Here, terminal differentiation sees no neural specific gene expression as compared to selective expression seen in neuroepithelia. So it would be possible for REST to mediate this repression by recruiting mSIN3B_{LF} or perhaps mSIN3A with associated de-acetylase activity and impose quiescent chromatin permanently over all RE1 containing genes.

Another possibility, though not mutually exclusive to the above, is that the SIN3 complex recruited by REST in mammalian cells does contain de-acetylase activity as in yeast. However, the substrate for de-acetylation and therefore acetylation is not histones. Close analysis of REST primary structure revealed a region from residue 440 to 600 which was 25% lysine. A protein database search (Swissprot) with this region showed very significant homology to histone1A and histone1B. This suggested to me that although H1A and H1B are not known to be targets for acetylation, that maybe REST may possess certain histone like properties, modification by acetylation being an example. The idea that REST may be subject to the activities of acetylase and de-acetylase

activity was further supported by discovering that in yeast, REST has a requirement for SIN3 to mediate repression. REST being the target for acetylation/de-acetylation would allow the unification of the observations that REST requires SIN3 and thus, in yeast de-acetylation and yet can mediate repression in transient transfection assays, presumably in the absence of histone substrate.

The idea that REST may be a target for acetylation can possibly explain the anomalous low electrophoretic mobility that recombinant REST elicits. The predicted molecular weight of REST based on primary structure is 116KDa and is the observed molecular weight for cellular REST. However, recombinant REST expressed either in cell lines (Chong *et al.*,1995, Tapia-Ramirez *et al.*,1997) or yeast (personal observation) elicits an apparent molecular weight of 200KDa. This slow mobility may be due the very lysine rich, and thus positively charged region mentioned above. The effect of charge on mobility would be analogous to the high mobility of HMG proteins due to an abundance of negatively charged residues. The fact that only recombinant REST runs at a high molecular weight may be due to over-expression and thus quenching of acetylase activity; acetylation would remove the positive charge at the ϵ -amino group and allow migration at the expected rate.

Recently, Gu and Roeder showed that p53 activity was modulated in a positive manner by acetylation of p53 by CBP/p300(Gu and Roeder,1997). The acetylation motifs in p53, LK(S/T)KKG and S(R/K)HKK are found at the acetylation sites for histone H3 and H4. REST yields 474-SKEKKP-479 and 513-SKTKKS-518 which conforms to the consensus (R/K)XKK where X is a negatively charged residue. Both of these sequences are identical/conserved between mouse and human REST

with diverged residues flanking either side and are within the lysine rich region described earlier.

A Model of REST Mediated Repression.

The exciting possibility that REST may be regulated by acetylation has inspired the following model for REST mediated repression: Based on the mode of p53 regulation, I propose that REST may exist in an inactive/non-repressing un-acetylated state. An acetylase, modifies the protein to yield an acetylated, active form. This would be consistent with endogenous REST, presumably active, running at 116KDa. This suggests that the bulk of recombinant REST is inactive due to lack of acetylation, and which therefore runs at 200KDa (see above). Active REST then recruits a SIN3 complex to target promoters containing N-CoR or N-CoR/mRPD3 in neuroepithelia and non-neuronal tissue respectively. Repression of neuronal genes in neuroepithelia is mediated by N-CoR and the N-terminal repression domain by interfering with the pre-initiation complex of target promoters. Repression in non-neuronal tissue is mediated by recruited mRPD3 as part of the SIN3 complex along with N-CoR which may also repress target promoters. This model takes into account the anomalous mobility of recombinant REST by invoking quenching of acetylase activity and is consistent with there being an acetylation motif like sequence in REST in a region that shares very significant homology with histones. Further, it goes some way to explaining how SIN3 is required by a repressor that functions in transient transfection assays: SIN3 may work on REST as part of a de-acetylase complex but may also, depending on the mSIN3B isoform, act at the level of core complex interactions in the case of neuroepithelia or at the level of chromatin remodelling in the case of non-neuronal tissue. Further, it would allow for REST mediated

repression in neuroepithelia of a subset of RE1 containing genes by selective use of REST repressible and REST 'immune' core promoter complexes. At the same time it would allow a single repression mechanism to silence all neuron specific genes in non-neuronal tissue by induction of quiescent chromatin.

FUTURE DIRECTIONS

The establishment of a yeast model in order to recapitulate REST repression in a genomic system more amenable to manipulation has allowed the establishment of a genetic link between REST and ySIN3. The emphasis should now be the verification of this link in mammalian systems.

Yeast SIN3 in all cases encountered so far is always associated with de-acetylase activity (see 'Introduction' and 'Chapter 5'). Rundlett *et al* have isolated a number of yeast histone de-acetylases (HDA1, RPD3, HOS1, HOS2, and HOS3) and show that they can be divided into trichostatin-A sensitive and trichostatin-A insensitive where trichostatin-A is a histone de-acetylase inhibitor (Rundlett *et al.*,1996). The sequential deletion of each of these genes, a rapid process using homologous recombination in yeast, should allow specificity of REST mediated de-acetylation in yeast to be established. Because RPD3 is over 78% similar to mammalian HD1 it is likely that RPD3 is the yeast homologue of mammalian RPD3. The above experiment will thus aid the identification of which mammalian de-acetylase complex(es) is used by REST in mammals.

In parallel, identification of which form(s) of mammalian SIN3 REST acts through may be established in yeast. The experiments outlined at the end of 'Chapter 5' involving the expression of

mSIN3A/GAL4 Activation Domain and mSIN3B/GAL4 Activation Domain fusions in yeast to convert REST to an activator would allow specificity of REST interaction with mammalian SIN3 isoforms to be established. This, in conjunction with the targeted antisense knock-out using phosphothiolated oligonucleotides to the SIN3 isoforms in the cell line 3T3 would allow the verification of any yeast derived specificity arguments in a mammalian setting. Further, analysis of endogenous gene expression by RNase protection and quantitative RT-PCR in 3T3 cells transfected with antisense oligonucleotides should be performed. Comparison of such data with expression of transiently transfected promoter constructs would allow questions of whether transiently transfected plasmids are 'naked' as suggested by Archer *et al* (Archer *et al.*,1992) or packaged in a form amenable to histone modification and subsequent regulation or whether other targets for acetylase/de-acetylase activity exists such as REST (see above) or perhaps the TFIID complex itself. Indeed, the TFIID complex has been suggested to mimic a histone octamer like structure and many of it's components e.g. TAF_{II}250 do possess intrinsic acetylase activity.

Another approach would be to use trapoxin and trichostatin-A on cell lines in parallel to the use on antisense oligonucleotides to help distinguish between which acetylase/de-acetylase activities are utilised by REST.

Upon identification of the RE1 sequence as an important regulatory element in the expression of the rat m4 gene, I have generated a model system in yeast to analyse the molecular events that belie REST mediated repression. A targeted approach in yeast to isolate

genes important in the REST repression pathway has identified SIN3 as essential to REST mediated repression. The use of this work and the experiments outlined above should contribute to the illumination of the molecular mechanisms that generate neuronal phenotypes in particular and cellular phenotypes in general.

REFERENCES.

REFERENCES

Alland, L., Muhle, R., Hou, H., Jr., Potes, J., Chin, L., Schreiber-Agus, N. and DePinho, R. A. (1997). "Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression [see comments]." Nature **387**(6628): 49-55.

Alonso, M. C. and Cabrera, C. V. (1988). "The achaete-scute gene complex of *Drosophila melanogaster* comprises four homologous genes." EMBO J **7**(8): 2585-2591.

Archer, T. K., Lefebvre, P., Wolford, R. G. and Hager, G. L. (1992). "Transcription factor loading on the MMTV promoter: a bimodal mechanism for promoter activation [published erratum appears in Science 1992 Apr 10;256(5054):161]." Science **255**(5051): 1573-1576.

Arias, J., Alberts, A. S., Brindle, P., Claret, F. X., Smeal, T., Karin, M., Feramisco, J. and Montminy, M. (1994). "Activation of cAMP and mitogen responsive genes relies on a common nuclear factor [see comments]." Nature **370**(6486): 226-229.

Ayer, D. E. and Eisenman, R. N. (1993). "A switch from Myc:Max to Mad:Max heterocomplexes accompanies monocyte/macrophage differentiation." Genes Dev **7**(11): 2110-2119.

Ayer, D. E., Laherty, C. D., Lawrence, Q. A., Armstrong, A. P. and Eisenman, R. N. (1996). "Mad proteins contain a dominant transcription repression domain." Mol Cell Biol **16**(10): 5772-5781.

Ayer, D. E., Lawrence, Q. A. and Eisenman, R. N. (1995). "Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3." Cell **80**(5): 767-776.

Banerji, J., Rusconi, S. and Schaffner, W. (1981). "Expression of a beta-globin gene is enhanced by remote SV40 DNA sequences." Cell **27**(2 Pt 1): 299-308.

Bannister, A. J. and Kouzarides, T. (1996). "The CBP co-activator is a histone acetyltransferase." Nature **384**(6610): 641-643.

Barberis, A., Pearlberg, J., Simkovich, N., Farrell, S., Reinagel, P., Bamdad, C., Sigal, G. and Ptashne, M. (1995). "Contact with a component of the polymerase II holoenzyme suffices for gene activation." Cell **81**(3): 359-368.

Bessis, A., Champiaux, N., Chatelin, L. and Changeux, J. P. (1997). "The neuron-restrictive silencer element: a dual enhancer/silencer crucial for patterned expression of a nicotinic receptor gene in the brain." Proc Natl Acad Sci U S A **94**(11): 5906-5911.

Bhattacharya, S., Eckner, R., Grossman, S., Oldread, E., Arany, Z., D'Andrea, A. and Livingston, D. M. (1996). "Cooperation of Stat2 and p300/CBP in signalling induced by interferon-alpha." Nature **383**(6598): 344-347.

Bonner, T. I., Buckley, N. J., Young, A. C. and Brann, M. R. (1987). "Identification of a family of muscarinic acetylcholine receptor genes." Science **237**: 527-532.

Bonner, T. I., Young, A. C., Brann, M. R. and Buckley, N.J. (1988). "Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes." Neuron **1**: 403-410.

Brown, D. A., Buckley, N. J., Caulfield, M. C., Duffy, S. M., Jones, S., Lamas, J. A., Marsh, S. J., Robbins, J. and Selyanko, A. A. (1995). Coupling of muscarinic acetylcholine receptors (mAChRs) to neural ion channels: closure of some K⁺ channels. Molecular mechanisms of muscarinic acetylcholine function Ed. J.Wess. Georgetown, TX, Landes,.

Brownell, J. E. and Allis, C. D. (1996). "Special HATs for special occasions: linking histone acetylation to chromatin assembly and gene activation." Curr Opin Genet Dev **6**(2): 176-184.

Buckley, N. J., Bonner, T. I. and Brann, M. R. (1988). "Localisation of a family of muscarinic receptor genes in rat brain." Journal of Neuroscience **8**: 4646-4652.

Buckley, N. J., Bonner, T. I., Buckley, C. M. and Brann, M. R. (1989). "Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells." Molecular Pharmacology **35**: 469-476.

Carlson, M., Osmond, B. C., Neigeborn, L. and Botstein, D. (1984). "A suppressor of SNF1 mutations causes constitutive high-level invertase synthesis in yeast." Genetics **107**(1): 19-32.

Caudy, M., Vassin, H., Brand, M., Tuma, R., Jan, L. Y. and Jan, Y. N. (1988). "daughterless, a Drosophila gene essential for both neurogenesis and sex determination, has sequence similarities to myc and the achaete-scute complex." Cell **55**(6): 1061-1067.

Cavallini, B., Faus, I., Matthes, H., Chipoulet, J., Winsor, B., Egly, J. and Chambon, P. (1989). "Cloning of the gene encoding the yeast protein BTF1Y, which can substitute for the human TATA box-binding factor." Proc. Natl. Acad. Sci. U.S.A **86**(24): 9803-9807.

Celenza, J. L. and Carlson, M. (1984). "Cloning and genetic mapping of SNF1, a gene required for expression of glucose-repressible genes in *Saccharomyces cerevisiae*." Mol. Cell. Biol. **4**(1): 49-53.

Celenza, J. L. and Carlson, M. (1986). "A yeast gene that is essential for release from glucose repression encodes a protein kinase." Science **233**(4769): 1175-1180.

Chasman, D. I. and Kornberg, R. D. (1990). "GAL4 protein: purification, association with GAL80 protein, and conserved domain structure." Mol. Cell. Biol. **10**(6): 2916-2923.

Chatterjee, S. and Struhl, K. (1995). "Connecting a promoter-bound protein to TBP bypasses the need for a transcriptional activation domain." Nature **374**: 820-821.

Chen, W. and Struhl, K. (1985). "Yeast mRNA initiation sites are determined primarily by specific sequences, not by the distance from the TATA element." EMBO J **4**(12): 3273-3280.

Chen, W. and Struhl, K. (1988). "Saturation mutagenesis of a yeast his3 "TATA element": genetic evidence for a specific TATA-binding protein." Proc. Natl. Acad. Sci. U.S.A **85**(8): 2691-2695.

Chong, J. A., Tapia-Ramirez, J., Kim, S., Toledo-Aral, J. J., Zheng, Y., Boutros, M. C., Altshuler, Y. M., Frohman, M. A., Kraner, S. D. and Mandel, G. (1995). "REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons." Cell **80**(6): 949-957.

Clark-Adams, C. D., Norris, D., Osley, M. A., Fassler, J. S. and Winston, F. (1988). "Changes in histone gene dosage alter transcription in yeast." Genes & Dev. **2**(2): 150-159.

Doi, R. H. and Wang, L.-F. (1986). "Multiple prokaryotic RNA polymerase sigma factors." Microbiol. Rev. **50**: 227-243.

Drapkin, R. and Reinberg, D. (1994). "The multifunctional TFIID complex and transcriptional control." Trends Biochem Sci **19**(11): 504-508.

Dynan, W. and Tjian, R. (1983). "Isolation of transcription factors that discriminate between different promoters recognized by RNA polymerase II." Cell **32**(3): 669-680.

Dynan, W. and Tjian, R. (1983). "The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter." Cell **35**(1): 79-87.

Dynan, W. and Tjian, R. (1985). "Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins." Nature **316**(6031): 774-778.

Dynlacht, B. D., Hoey, T. and Tjian, R. (1991). "Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation." Cell **55**: 563.

- Ellis, H. M., Spann, D. R. and Posakony, J. W. (1990). "extramacrochaetae, a negative regulator of sensory organ development in *Drosophila*, defines a new class of helix-loop-helix proteins." Cell **61**(1): 27-38.
- Errington, J. (1993). "B.subtilis sporulation:regulation of gene expression and control of morphogenesis." Microbiol. Rev. **57**: 1-33.
- Estruch, F. and Carlson, M. (1990). "SNF6 encodes a nuclear protein that is required for expression of many genes in *Saccharomyces cerevisiae*." Mol. Cell. Biol. **10**(6): 2544-2553.
- Farrell, S., Simkovich, N., Wu, Y., Barberis, A. and Ptashne, M. (1996). "Gene activation by recruitment of the RNA polymerase II holoenzyme." Genes & Dev. **10**(18): 2359-2367.
- Frasch, M. and Levine, M. (1987). "Complementary patterns of even-skipped and fushi tarazu expression involve their differential regulation by a common set of segmentation genes in *Drosophila*." Genes & Dev. **1**(9): 981-995.
- Garrell, J. and Modolell, J. (1990). "The *Drosophila* extramacrochaetae locus, an antagonist of proneural genes that, like these genes, encodes a helix-loop-helix protein." Cell **61**(1): 39-48.
- Gerber, H. P., Seipel, K., Georgiev, O., Hofferer, M., Hug, M., Rusconi, S. and Schaffner, W. (1994). "Transcriptional activation modulated by homopolymeric glutamine and proline stretches." Science **263**: 808-810.
- Gill, G., Pascal, E., Tseng, Z. H. and Tjian, R. (1991). "A glutamine-rich hydrophobic patch in transcription factor Sp1 contacts the dTAFII110 component of the *drosophila* TFIID complex and mediates transcriptional activation." Proc. Natl. Acad. Sci U.S.A **91**: 192-196.
- Goebel, M. and Yanagida, M. (1991). "The TPR snap helix: a novel protein repeat motif from mitosis to transcription." Trends Biochem Sci **16**(5): 173-177.

Goto, T., Macdonald, P. and Maniatis, T. (1989). "Early and late periodic patterns of even skipped expression are controlled by distinct regulatory elements that respond to different spatial cues." Cell **57**(3): 413-422.

Gu, W. and Roeder, R. G. (1997). "Activation of p53 Sequence-Specific DNA Binding by Acetylation of the p53 c-terminal Domain." Cell **90**: 595-605.

Ha, I., Roberts, S., Maldonado, E., Sun, X., Kim, L. U., Green, M. and Reinberg, D. (1993). "Multiple functional domains of human transcription factor IIB: distinct interactions with two general transcription factors and RNA polymerase II." Genes & Dev. **7**(6): 1021-1032.

Hahn, S., Buratowski, S., Sharp, P. A. and Guarente, L. (1989). "Isolation of the gene encoding the yeast TATA binding protein TFIID: a gene identical to the SPT15 suppressor of Ty element insertions." Cell **58**(6): 1173-1181.

Haldenwang, W. G. (1995). "The sigma factors of B.subtilis." Microbiol. Rev. **59**: 1-30.

Haldenwang, W. G., Lang, N. and Losick, R. (1981). "A sporulation-induced sigma-like regulatory protein from B.subtilis." Cell **23**: 615-624.

Haldenwang, W. G. and Losick, R. (1980). "A novel RNA polymerase sigma factor from B.subtilis." Proc.Nat.Acad.Sci.USA **77**: 7000-7004.

Halford, S., Wadey, R., Roberts, C., Daw, S. C., Whiting, J. A., O'Donnell, H., Dunham, I., Bentley, D., Lindsay, E., Baldini, A. and et, a. (1993). "Isolation of a putative transcriptional regulator from the region of 22q11 deleted in DiGeorge syndrome, Shprintzen syndrome and familial congenital heart disease." Hum. Mol. Genet. **2**(12): 2099-2107.

Harding, K., Hoey, T., Warrior, R. and Levine, M. (1989). "Autoregulatory and gap gene response elements of the even-skipped promoter of Drosophila." EMBO J **8**(4): 1205-1212.

Hassal, C. J. S., Stanford, S. C., Burnstock, G. and Buckley, N. J. (1993). "Co-expression of four muscarinic receptor genes by the intrinsic neurons of the rat and guinea-pig heart." Neuroscience **56**: 1041-1048.

Heery, D. M., Kalkhoven, E., Hoare, S. and Parker, M. G. (1997). "A signature motif in transcriptional co-activators mediates binding to nuclear receptors [see comments]." Nature **387**(6634): 733-736.

Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenman, R. N., Rose, D. W., Glass, C. K. and Rosenfeld, M. G. (1997). "A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression [see comments]." Nature **387**(6628): 43-48.

Henriksson, M. and Luscher, B. (1996). "Proteins of the Myc network: essential regulators of cell growth and differentiation." Adv. Cancer Res. **68**: 109-182.

Herschbach, B. M., Arnaud, M. B. and Johnson, A. D. (1994). "Transcriptional repression directed by the yeast alpha 2 protein in vitro." Nature **370**(6487): 309-311.

Hirano, T., Kinoshita, N., Morikawa, K. and Yanagida, M. (1990). "Snap helix with knob and hole: essential repeats in *S. pombe* nuclear protein nuc2+." Cell **60**(2): 319-328.

Hoey, T., Weinzierl, R., Gill, G., Chen, J. L., Dynlacht, B. D. and Tjian, R. (1993). "Molecular cloning and functional analysis of drosophila TAF110 reveal properties expected of coactivators." Cell **46**: 247.

Hope, I. A. and Struhl, K. (1986). "Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast." Cell **46**: 885-894

Horikoshi, M., Wang, C. K., Fujii, H., Cromlish, J. A., Weil, P. A. and Roeder, R. G. (1989). "Cloning and structure of a yeast gene encoding a general transcription initiation factor TFIID that binds to the TATA box." Nature **341**(6240): 299-303.

Howland, D. S., Hemmendinger, L. M., Carroll, P. D., Estes, P. S., Melloni, R. H. J. and DeGennaro, L. J. (1991). "Positive- and negative-acting promoter sequences regulate cell type-specific expression of the rat synapsin I gene." Brain.Res.Mol.Brain.Res. **11**(3-4): 345-353.

Hurlin, P. J., Foley, K. P., Ayer, D. E., Eisenman, R. N., Hanahan, D. and Arbeit, J. M. (1995). "Regulation of Myc and Mad during epidermal differentiation and HPV-associated tumorigenesis." Oncogene **11**(12): 2487-2501.

Ishiguro, H., Kim, K. T., Joh, T. H. and Kim, K. S. (1993). "Neuron-specific expression of the human dopamine beta-hydroxylase gene requires both the cAMP-response element and a silencer region." J. Biol. Chem. **268**(24): 17987-17994.

Jackson, P., McDonald, J., Lees-Miller, S. and Tjian, R. (1990). "GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase." Cell **63**: 155-165.

Jacob, F. and Monod, J. (1961). "Genetic regulatory mechanisms in the synthesis of proteins." J. Mol. Biol. **3**: 318-356.

Johnston, M. (1987). "A model fungal gene regulatory mechanism: the GAL genes of *Saccharomyces cerevisiae*." Microbiol. rev. **51**: 458-476.

Johnston, M., Flick, J. S. and Pexton, T. (1994). "Multiple mechanisms provide rapid and stringent glucose repression of GAL gene expression in *Saccharomyces cerevisiae*." Mol. Cell. Biol. **14**(6): 3834-3841.

Johnston, S. A., Salmeron, S. M. and Dincher, S. S. (1987). "Interaction of positive and negative regulatory proteins in the galactose regulon of yeast." Cell **50**: 143-146.

Julien, J., Beaudet, L., Tretjakoff, I. and Peterson, A. (1990). "Neurofilament gene expression in transgenic mice." J. Physiol. (Paris) **84**: 50-52.

Kadosh, D. and Struhl, K. (1997). "Repression by Ume6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters." Cell **89**(3): 365-371.

Kakkis, E. and Calame, K. (1987). "A plasmacytoma-specific factor binds the c-myc promoter region." Proc. Natl. Acad. Sci. USA **84**(20): 7031-7035.

Kakkis, E., Riggs, K. J., Gillespie, W. and Calame, K. (1989). "A transcriptional repressor of c-myc." Nature **339**(6227): 718-721.

Kasten, M., Ayer, D. E. and Stillman, D. J. (1996). "SIN3-Dependent Transcriptional Repression by interaction with the Mad1 DNA-Binding Protein." Mol. Cell. Biol. **16**(8): 4215-4221.

Keleher, C. A., Redd, M. J., Schultz, J., Carlson, M. and Johnson, A. D. (1992). "Ssn6-Tup1 is a general repressor of transcription in yeast." Cell **68**(4): 709-719.

Kidd, S. (1992). "Characterization of the *Drosophila* cactus locus and analysis of interactions between cactus and dorsal proteins." Cell **71**(4): 623-635.

Kim, S., Na, J., Hampsey, M. and Reinberg, D. (1997). "The Dr1/DRAP1 heterodimer is a global repressor of transcription in vivo." Proc. Natl. Acad. Sci. U.S.A. **94**(3): 820-825.

Kim, T. K., Hashimoto, S., Kelleher, R. J., Flanagan, P. M., Kornberg, R. D., Horikoshi, M. and Roeder, R. G. (1994). "Effects of activation-defective TBP mutations on transcription initiation in yeast." Nature **369**: 6477.

Klages, N. and Strubin, M. (1995). "Stimulation of RNA polymerase II transcription initiation by recruitment of TBP in vivo." Nature **374**(6525): 822-823.

Klarsfeld, A., Bessereau, J. L., Salmon, A. M., Triller, A., Babinet, C. and Changeux, J. P. (1991). "An acetylcholine receptor alpha-subunit

promoter conferring preferential synaptic expression in muscle of transgenic mice." EMBO J **10**(3): 625-632.

Klein, C. and Sruhl, K. (1994). "Increased Recruitment of TATA-Binding Protein by Transcriptional Activation Domains in Vivo." Science **266**: 280-282.

Koleske, A. J. and Young, R. A. (1994). "An RNA polymerase holoenzyme responsive to activators." Nature **368**: 466-469.

Kruger, W. and Herskowitz, I. (1991). "A negative regulator of HO transcription, SIN1 (SPT2), is a nonspecific DNA-binding protein related to HMG1." Mol. Cell. Biol. **11**(8): 4135-4146.

Kubo, T., Fukuda, K., Mikami, A., Maeda, A., Takahashi, H., Mishina, M., Haga, T., Haga, K., Iciyama, A., Kangawa, K., Kojima, M., Matsuo, M., Hirose, T. and Numa, S. (1987). "Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor." Nature **323**: 411-416.

Kuchin, S., Yeghiayan, P. and Carlson, M. (1995). "Cyclin-dependent protein kinase and cyclin homologs SSN3 and SSN8 contribute to transcriptional control in yeast." Proc. Natl. Acad. Sci. USA **92**(9): 4006-4010.

Kwok, R. P., Lundblad, J. R., Chrivia, J. C., Richards, J. P., Bachinger, H. P., Brennan, R. G., Roberts, S. G., Green, M. R. and Goodman, R. H. (1994). "Nuclear protein CBP is a coactivator for the transcription factor CREB [see comments]." Nature **370**(6486): 223-226.

Laurent, B. C., Treitel, M. A. and Carlson, M. (1991). "Functional interdependence of the yeast SNF2, SNF5, and SNF6 proteins in transcriptional activation." Proc. Natl. Acad. Sci. USA **88**(7): 2687-2691.

Lazareno, S., Buckley, N. J. and Roberts, F. F. (1990). "Characterisation of muscarinic M4 binding site in rabbit lung, chicken heart and NG108-15 cells." Molecular Pharmacology **38**: 805-815.

Lee, N., Wilcox, G., Gielow, W., Arnold, J., Cleary, P. and Englesberg, E. (1974). "In vitro activation of the transcription of araBAD operon by araC activator." Proc. Natl. Acad. Sci. USA **71**(3): 634-638.

Lehming, N., Thanos, D., Brickman, J. M., Ma, J., Maniatis, T. and Ptashne, M. (1994). "An HMG-like protein that can switch a transcriptional activator to a repressor." Nature **371**(6493): 175-179.

Leuther, K. K. and Johnston, S. A. (1992). "Nondissociation of GAL4 and GAL80 in vivo after galactose induction." Science **256**(5061): 1333-1335.

Leuther, K. K., Salmeron, J. M. and Johnson, S. A. (1993). "Genetic evidence that an activation domain of GAL4 does not require acidity and may form a β sheet." Cell **72**: 575-585.

Lewin, B. (1997). GENE VI. New York, Oxford University Press.

Li, L., Suzuki, T., Mori, N. and Greengard, P. (1993). "Identification of a functional silencer element involved in neuron-specific expression of the synapsin I gene." Proc. Natl. Acad. Sci. U.S.A **90**(4): 1460-1464.

Liao, S. M., Zhang, J., Jeffery, D. A., Koleske, A. J., Thompson, C. M., Chao, D. M., Viljoen, M., van-Vuuren, H. J. and Young, R. A. (1995). "A kinase-cyclin pair in the RNA polymerase II holoenzyme [see comments]." Nature **374**(6518): 193-196.

Liu, J., Wilson, T. E., Milbrandt, J. and Johnston, M. (1993). "Identifying DNA binding sites and analyzing DNA-binding domains using a yeast selection system." METHODS: A Companion to Methods in Enzymology. **5**: 125-137.

Lonnerberg, P., Schoenherr, C. J., Anderson, D. J. and Ibanez, C. F. (1996). "Cell Type-specific regulation of Choline Acetyltransferase Gene Expression." J. Biol. Chem **271**(52): 33358-33365.

Losick, R. and Stragier, P. (1992). "Crisscross regulation of cell-type-specific gene expression during development in *B. subtilis*." Nature **355**(6361): 601-604.

Losick, R., Youngman, P. and Piggot, P. J. (1986). "Genetics of endospore formation in *B. subtilis*." Ann. Rev. Genet. **20**: 625-669.

Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F. and Richmond, T. J. (1997). "Crystal structure of the nucleosome core particle at 2.8 Angstrom resolution." Nature **389**: 251-260.

Lutfiyya, L. L. and Ohnston, M. (1996). "Two Zinc-Finger-Repressors Are Responsible for Repression of SUC2 Expression." Mol. Cell. Biol. **16**(9): 4790-4797.

Ma, J. and Ptashne, M. (1987). "The carboxy-terminal 30 amino acids of gal4 are recognised by gal80." Cell **50**: 137-142.

Ma, J. and Ptashne, M. (1987). "Deletion analysis of GAL4 defines two transcriptional activating segments." Cell **48**: 847-853.

MacDonald, G. H., Itoh-Lindstrom, Y. and Ting, J. P. (1995). "The transcriptional regulatory protein, YB-1, promotes single-stranded regions in the DRA promoter." J. Biol. Chem. **270**(8): 3527-3533.

Mak, J. C., Haddad, E. B., Buckley, N. J. and Barnes, P. J. (1993). "Visualisation of muscarinic m4 mRNA and M4 receptor subtype in rabbit lung." Life Sciences **53**: 1501-1508.

Marin, M., Karis, A., Visser, P., Grosveld, F. and Philipsen, S. (1997). "Transcription Factor Sp1 Is Essential for Early Embryonic Development but Dispensable for Cell Growth and Differentiation." Cell **89**: 619-628.

Maue, R. A., Kraner, S. D., Goodman, R. H. and Mandel, G. (1990). "Neuron-specific expression of the rat brain type II sodium channel is directed by upstream regulatory elements." Neuron **4**: 223-231.

Maxon, M. E., Goodrich, J. A. and Tjian, R. (1994). "Transcription factor IIE binds preferentially to RNA polymerase IIa and recruits TFIID: a model for promoter clearance." Genes & Dev. **8**(5): 515-524.

Mieda, M., Haga, T. and Saffen, T. W. (1996). "Promoter Region of the Rat m4 Muscarinic Receptor Gene Contains a Cell Type-Specific Silencer Element." J.Biol.Chem. **271**(9): 5177-5182.

Moqtaderi, Z., Bai, Y., Poon, D., Weil, P. A. and Struhl, K. (1996). "TBP-associated factors are not generally required for transcriptional activation in yeast [see comments]." Nature **383**(6596): 188-191.

Moqtaderi, Z., Yale, J., Struhl, K. and Buratowski, S. (1996). "Yeast homologues of higher eukaryotic TFIID subunits." Proc. Natl. Acad. Sci. U.S.A. **93**(25): 14654-14658.

Mori, N., Stein, R., Sigmund, D. and Anderson, D. J. (1990). "A cell type-preferred silencer element that controls the neural specific expression of the SCG10 gene." Neuron **4**: 583-594.

Murre, C., McCaw, P. S. and Baltimore, D. (1989). "A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins." Cell **56**(5): 777-783.

Mylin, L. M., Bhat, J. P. and Hopper, J. E. (1989). "Regulated phosphorylation and dephosphorylation of GAL4, a transcriptional activator." Genes & Dev. **3**(8): 1157-1165.

Mylin, L. M., Johnston, M. and Hopper, J. E. (1990). "Phosphorylated forms of GAL4 are correlated with ability to activate transcription." Mol. Cell. Biol. **10**(9): 4623-4629.

Nasmyth, K. (1983). "Molecular analysis of a cell lineage." Nature **302**(5910): 670-676.

Nasmyth, K., Stillman, D. and Kipling, D. (1987). "Both positive and negative regulators of HO transcription are required for mother-cell-specific mating-type switching in yeast." Cell **48**(4): 579-587.

Nehlin, J. O. and Ronne, H. (1990). "Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumour finger proteins." EMBO J **9**(9): 2891-2898.

Neigeborn, L. and Carlson, M. (1984). "Genes affecting the regulation of SUC2 gene expression by glucose repression in *Saccharomyces cerevisiae*." Genetics **108**(4): 845-858.

Neigeborn, L., Rubin, K. and Carlson, M. (1986). "Suppressors of SNF2 mutations restore invertase derepression and cause temperature-sensitive lethality in yeast." Genetics **112**(4): 741-753.

Ninfa, A. J., Reitzer, L. J. and Magasanik, B. (1987). "Initiation of transcription at the bacterial *glnAp2* promoter by purified *E. coli* components is facilitated by enhancers." Cell **50**(7): 1039-1046.

Parthun, M. R. and Jaehning, J. A. (1992). "A transcriptionally active form of GAL4 is phosphorylated and associated with GAL80." Mol. Cell. Biol. **12**(11): 4981-4987.

Pathak, B. G., Nuemann, J. C., Croyle, M. L. and Lingrel, J. B. (1994). "The presence of both negative and positive elements in the 5'-flanking sequence of the rat Na,K-ATPase alpha3 subunit gene are required for brain expression in transgenic mice." Nuc. Acids Res. **22**(22): 4748-4755.

Pazin, M. J. and Kadonaga, J. T. (1997). "What's up and down with histone deacetylation and transcription?" Cell **89**(3): 325-328.

Pengue, G. and Lania, L. (1996). "Kruppel-associated box-mediated repression of RNA polymerase II promoters is influenced by the arrangement of basal promoter elements." Proc. Natl. Acad. Sci. U.S.A **93**(3): 1015-1020.

Peralta, E. G., Ashkenazi, A., Winslow, J. W., Smith, D. H., Ramachandran, J. and Capon, D. J. (1987). "Distinct primary structures, ligand binding

properties and tissue-specific expression of four human muscarinic acetylcholine receptors." EMBO J. **6**: 3923-3929.

Peter-Verrijzer, C., Chen, J.-L., Yokomori, K. and Tjian, R. (1995). "Binding of TAFs to core elements directs promoter selectivity by RNA polymerase II." Cell **81**: 1115-1125.

Peterson, C. L. and Herskowitz, I. (1992). "Characterization of the yeast SWI1, SWI2, and SWI3 genes, which encode a global activator of transcription." Cell **68**(3): 573-583.

Peterson, C. L. and Tamkun, J. W. (1995). "The SWI-SNF complex: a chromatin remodeling machine?" Trends Biochem. Sci. **20**(4): 143-146.

Ptashne, M. and Gann, A. (1997). "Transcriptional Activation By Recruitment." Nature **386**: 569-577.

Pugh, B. F. and Tjian, R. (1991). "Transcription from a TATA-less promoter requires a multisubunit complex." Genes & Dev. **5**: 1935.

Redd, M. J., Arnaud, M. B. and Johnson, A. D. (1997). "A complex composed of tup1 and ssn6 represses transcription in vitro." J. Biol. Chem. **272**(17): 11193-11197.

Riggs, K. J., Merrell, K. T., Wilson, G. and Calame, K. (1991). "Common factor 1 is a transcriptional activator which binds in the c-myc promoter, the skeletal alpha-actin promoter, and the immunoglobulin heavy-chain enhancer." Mol. Cell. Biol. **11**(3): 1765-1769.

Roth, S., Hiromi, Y., Godt, D. and Nusslein-Volhard, C. (1991). "cactus, a maternal gene required for proper formation of the dorsoventral morphogen gradient in Drosophila embryos." Development **112**(2): 371-388.

Rundlett, S. E., Carmen, A. A., Kobayashi, R., Bavykin, S., Turner, B. M. and Grunstein, M. (1996). "HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription." Proc. Natl. Acad. Sci. USA **93**(25): 14503-14508.

Saha, S., Brickman, J. M., Lehming, N. and Ptashne, M. (1993). "New eukaryotic transcriptional repressors." Nature **363**(6430): 648-652.

Sakurai, H., Ohishi, T. and Fukasawa, T. (1994). "Two alternative pathways of transcription initiation in the yeast negative regulatory gene GAL80." Mol. Cell. Biol. **14**(10): 6819-6828.

Salmeron, J. M., Leuther, K. K. and Johnston, S. E. (1990). "GAL4 mutations that separate the transcriptional activation and Gal80 interactive functions of the yeast gal4 protein." Genetics **125**: 21-27.

Sasse-Dwight, S. and Gralla, J. D. (1988). "Probing the Escherichia coli glnALG upstream activation mechanism in vivo." Proc. Natl. Acad. Sci. USA **85**(23): 8934-8938.

Sauer, F., Fondell, J. D., Ohkuma, Y., Roeder, R. G. and Jackle, H. (1995). "Control of transcription by Kruppel through interactions with TFIIB and TFIIE beta [see comments]." Nature **375**(6527): 162-164.

Sauer, F. and Jackle, H. (1993). "Dimerization and the control of transcription by Kruppel." Nature **364**(6436): 454-457.

Schiestl, R. H. and Gietz, R. D. (1989). "High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier." Curr. Genet. **16**(5-6): 339-346.

Schmidt, M. C., Kao, C. C., Pei, R. and Berk, A. J. (1989). "Yeast TATA-box transcription factor gene." Proc. Natl. Acad. Sci. U.S.A **86**(20): 7785-7789.

Schoch, S., Cibelli, G. and Thiel, G. (1996). "Neuron-specific Gene Expression of Synapsin I." J. Biol. Chem. **271**(6): 3317-3323.

Schoenherr, C. J. and Anderson, D. J. (1995). "The Neuron-Restrictive Silencer Factor (NRSF): A Coordinate Repressor of Multiple Neuron-Specific Genes." Science **267**: 1360-1363.

Scholl, T., Stevens, M. B., Mahanta, S. and Strominger, J. L. (1996). "A Zinc Finger Protein That Represses Transcription of the Human MHC ClassII Gene, DPA." J. Immunology **156**: 1448-1457.

Schreiber-Agus, N., Chin, L., Chen, K., Torres, R., Rao, G., Guida, P., Skoultchi, A. I. and DePinho, R. A. (1995). "An amino-terminal domain of Mx11 mediates anti-Myc oncogenic activity and interacts with a homolog of the yeast transcriptional repressor SIN3." Cell **80**(5): 777-786.

Seipel, K., Georgiev, O. and Schaffner, W. (1994). "A minimal transcription activation domain consisting of a specific array of aspartic acid and leucine residues." Biol. Chem. Hoppe-Seyler **375**: 463-470.

Shimatake, H. and Rosenberg, M. (1981). "Purified lambda regulatory protein cII positively regulates promoters for lysogenic development." Nature **292**: 128-132.

Sikorski, R. S., Boguski, M. S., Goebel, M. and Hieter, P. (1990). "A repeating amino acid motif in CDC23 defines a family of proteins and a new relationship among genes required for mitosis and RNA synthesis." Cell **60**(2): 307-317.

Sikorski, R. S. and Heiter, P. (1989). "A system of shuttle vectors and yeast host strains designed for the efficient manipulation of DNA in *Saccharomyces cerevisiae*." Genetics **122**: 19-27.

Small, S., Kraut, R., Hoey, T., Warrior, R. and Levine, M. (1991). "Transcriptional regulation of a pair-rule stripe in *Drosophila*." Genes & Dev. **5**(5): 827-839.

Stanojevic, D., Hoey, T. and Levine, M. (1989). "Sequence-specific DNA-binding activities of the gap proteins encoded by hunchback and Kruppel in *Drosophila*." Nature **341**(6240): 331-335.

Stillman, D. J., Dorland, S. and Yu, Y. (1994). "Epistasis analysis of suppressor mutations that allow HO expression in the absence of the yeast SW15 transcriptional activator." Genetics **136**(3): 781-788.

Tamura, T., Sumita, K., Hirose, S. and Mikoshiba, K. (1990). "Core promoter of the mouse myelin basic protein gene governs brain-specific transcription in vitro." EMBO Journal **9**(10): 3101-3108.

Tanaka, M., Clouston, W. M. and Herr, W. (1994). "The OCT-2 glutamine-rich and proline-rich activation domains can synergise with each other or duplicates of themselves to activate transcription." Mol.Cell.Biol **14**: 6046-6055.

Tanaka, M. and Herr, W. (1994). "Reconstitution of transcriptional activation domains by reiteration of short peptide segments reveals the modular organisation of a glutamine-rich activation domain." Mol. Cell. Biol. **14**: 6056-6067.

Tanese, N., Pugh, B. f. and Tjian, R. (1991). "Coactivators for proline activator purified from the multisubunit human TFIID complex." Genes & Dev. **5**: 2212

Tapia-Ramirez, J., Eggen, B., Peral-Rubio, M., Toledo-Aral, J. and Mandel, G. (1997). "A single zinc finger motif in the silencing factor REST represses the neural-specific type II sodium channel promoter." Proc. Natl. Acad. Sci. U.S.A. **94**(4): 1177-1182.

Ting, J. P., Painter, A., Zeleznik-Le, N. J., MacDonald, G., Moore, T. M., Brown, A. and Schwartz, B. D. (1994). "YB-1 DNA-binding protein represses interferon gamma activation of class II major histocompatibility complex genes." J. Exp. Med **179**(5): 1605-1611.

Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K. and Rosenfeld, M. G. (1997). "The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function [see comments]." Nature **387**(6634): 677-684.

Travers, A. A. and Burgess, R. R. (1969). "Cyclic re-use of the RNA polymerase sigma factor." Nature **222**(193): 537-540.

Treital, M. A. and Carlson, M. (1995). "Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein." Proc. Natl. Acad. Sci. **92**: 3132-3136.

Trumbly, R. J. (1986). "Isolation of *Saccharomyces cerevisiae* mutants constitutive for invertase synthesis." J. Bacteriol **166**(3): 1123-1127.

Trumbly, R. J. (1992). "Glucose repression in the yeast *Saccharomyces cerevisiae*." Mol Microbiol **6**: 15-21.

Turner, B. M. (1993). "Decoding the nucleosome." Cell **75**(1): 5-8.

Usheva, A., Maldonado, E., Goldring, A., Lu, H., Houbavi, C., Reinberg, D. and Aloni, Y. (1992). "Specific interaction between the nonphosphorylated form of RNA polymerase II and the TATA-binding protein." Cell **69**(5): 871-881.

Van-Doren, M., Ellis, H. M. and Posakony, J. W. (1991). "The *Drosophila* extramacrochaetae protein antagonizes sequence-specific DNA binding by daughterless/achaete-scute protein complexes." Development **113**(1): 245-255.

Vidal, M. and Gaber, R. F. (1991). "RPD3 encodes a second factor required to achieve maximum positive and negative transcriptional states in *Saccharomyces cerevisiae*." Mol. Cell. Biol. **11**(12): 6317-6327.

Villares, R. and Cabrera, C. V. (1987). "The achaete-scute gene complex of *D. melanogaster*: conserved domains in a subset of genes required for neurogenesis and their homology to myc." Cell **50**(3): 415-424.

Vinals, F., Fandos, C., Santalucia, T., Ferre, J., Testar, X., Palacin, M. and Zorzano, A. (1997). "Myogenesis and MyoD Down-regulate Sp1." J. Biol. Chem **272**(20): 12913-12921.

Vindevoghel, L., Chung, K.-T., Davis, A., Kouba, D., Kivirikko, S., Alders, H., Uitto, J. and Mauviel, A. (1997). "A GT-rich Sequence Binding the Transcription Factor Sp1 Is Crucial for High Expression of the Human

Type VII Collagen Gene (COL7A1) in Fibroblasts and Keratinocytes.” J.Biol.Chem **272**(15): 10196-10204.

Voronova, A. and Baltimore, D. (1990). “Mutations that disrupt DNA binding and dimer formation in the E47 helix-loop-helix protein map to distinct domains.” Proc Natl Acad Sci U S A **87**(12): 4722-4726.

Wahi, M. and Johnson, A. D. (1995). “Identification of genes required for alpha 2 repression in *Saccharomyces cerevisiae*.” Genetics **140**(1): 79-90.

Walker, S. S., Reese, J. C., Apone, L. M. and Green, M. R. (1996). “Transcription activation in cells lacking TAFIIS [see comments].” Nature **383**(6596): 185-188.

Wang, G., Ide, K., Nukina, N., Goto, J., Ichikawa, Y., Uchida, K., Sakamoto, T. and Kanazawa, I. (1997). “Machado-Joseph disease gene product identified in lymphocytes and brain.” Biochem. Biophys. Res. Commun. **233**(2): 476-479.

Wang, H. and Stillman, D. J. (1993). “Transcriptional repression in *Saccharomyces cerevisiae* by a SIN3-LexA fusion protein.” Mol. Cell. Biol. **13**(3): 1805-1814.

Wang, R. and Reed, R. (1993). “Molecular cloning of the olfactory neuronal transcription factor Olf-1 by genetic selection in yeast.” Nature **364**(6433): 121-126.

Wang, X.-M., Tsay, H.-J. and Schmidt, J. (1990). “Expression of the acetylcholine receptor delta-subunit gene in differentiating chick muscle cells is activated by an element that contains two 16bp copies of a segment of the alpha-subunit gene.” EMBO Journal **9**(3): 783-790.

Weinzierl, R., Dynlacht, B. D. and Tjian, R. (1993). “Largest subunit of drosophila transcription factor IID directs assembly of a complex containing TBP and a coactivator.” Nature **362**: 511.

Winston, F. and Carlson, M. (1992). "Yeast SNF/SWI transcriptional activators and the SPT/SIN chromatin connection." Trends Genet. **8**(11): 387-391.

Wolffe, A. P. and Pruss, D. (1996). "Deviant nucleosomes: the functional specialization of chromatin." Trends Genet. **12**(2): 58-62.

Wood, I. C., Roopra, A. and Buckley, N. J. (1996). "Neural specific expression of the m4 muscarinic acetylcholine receptor gene is mediated by a RE1/NRSE-type silencing element." J. Biol. Chem. **271**(24): 14221-14225.

Wood, I. C., Roopra, A., Harrington, C. and Buckley, N. J. (1995). "Structure of the m4 cholinergic muscarinic receptor gene and its promoter." J. Biol. Chem. **270**:

Wu, Y., Reece, R. J. and Ptashne, M. (1996). "Quantitation of putative activator-target affinities predicts transcriptional activating potential." EMBO J **15**: 3951-3963.

Wuenschell, C.W., Mori, N., Anderson, D.J. (1990). "Analysis of SCG10 gene expression in transgenic mice reveals that neural specificity is achieved through selective derepression." Neuron **4**: 595-602

Xiao, H., Pearson, A., Coulombe, B., Truant, R., Zhang, S., Regier, J. L., Triezenberg, S. J., Reinberg, D., Flores, O., Ingles, C. J. and et, a. (1994). "Binding of basal transcription factor TFIID to the acidic activation domains of VP16 and p53." Mol. Cell. Biol. **14**(10): 7013-7024.

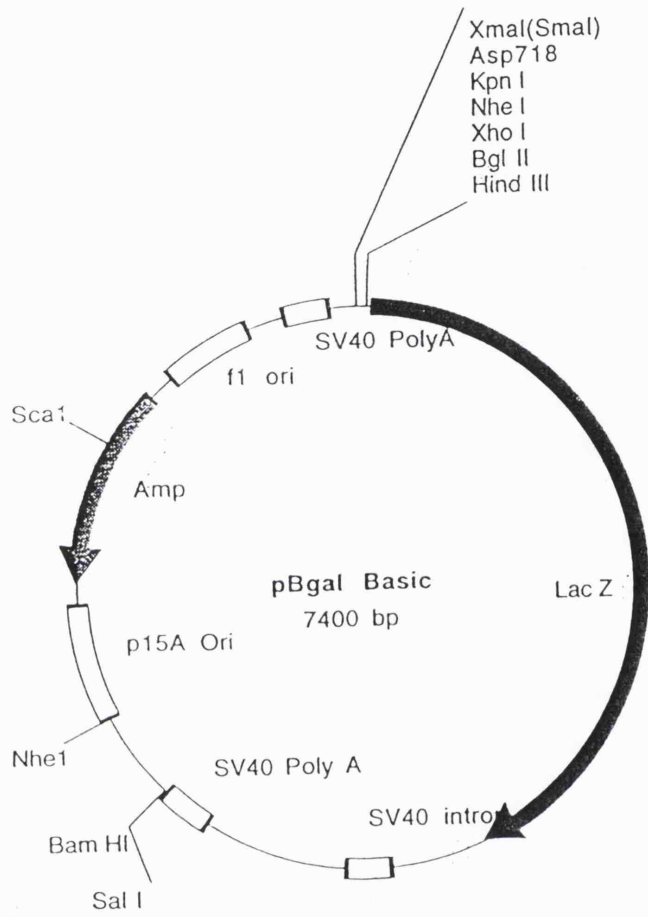
Yoshimoto, H. and Yamashita, I. (1991). "The GAM1/SNF2 gene of *Saccharomyces cerevisiae* encodes a highly charged nuclear protein required for transcription of the STA1 gene." Mol. Gen. Genet. **228**(1-2): 270-280.

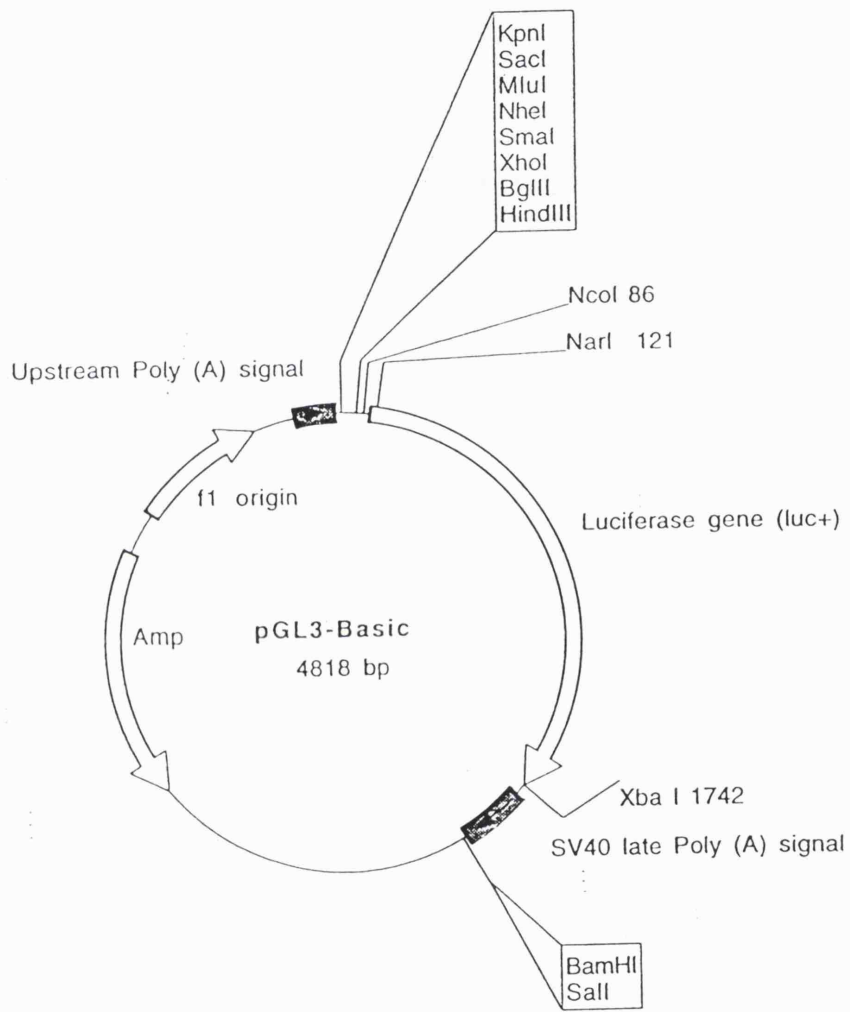
Yu, Y., Li, W., Su, K., Yussa, M., Han, W., Perrimon, N. and Pick, L. (1997). "The nuclear hormone receptor Ftz-F1 is a cofactor for the *Drosophila* homeodomain protein Ftz." Nature **385**(6616): 552-555.

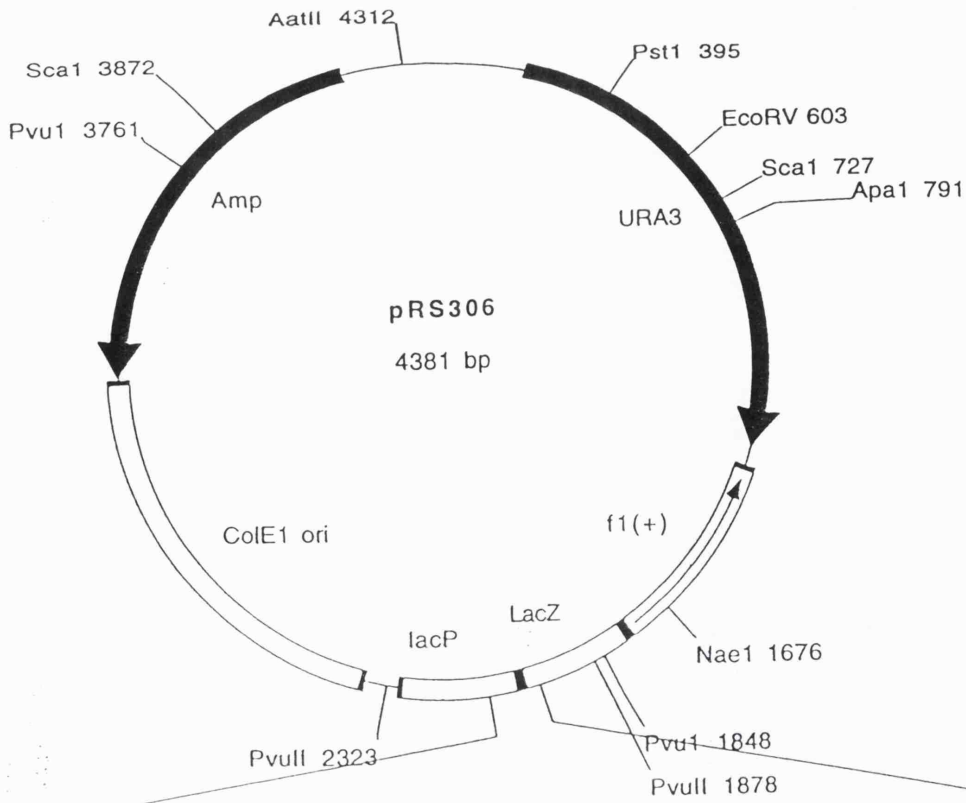
Zawel, L. and Reinberg, D. (1992). "Advances in RNA polymerase II transcription." Curr. Opin. Cell. Biol **4**: 488.

Zhang, J. J., Vinkemeier, U., Gu, W., Chakravarti, D., Horvath, C. M. and Darnell, J., Jr. (1996). "Two contact regions between Stat1 and CBP/p300 in interferon gamma signaling." Proc. Natl. Acad. Sci. USA **93**(26): 15092-15096.

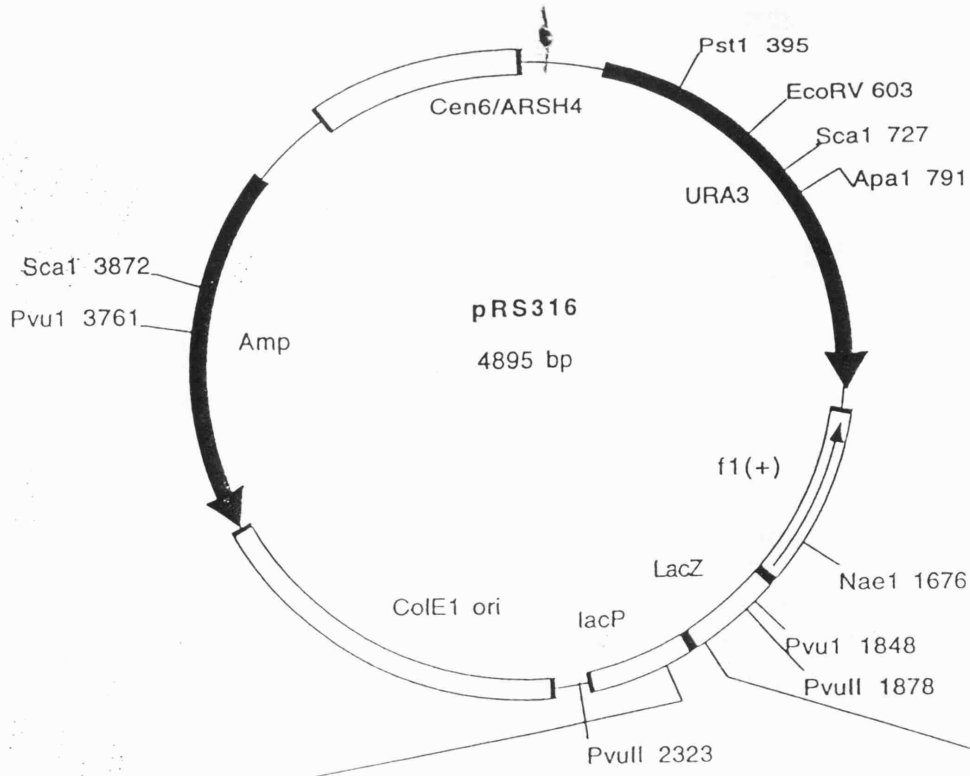
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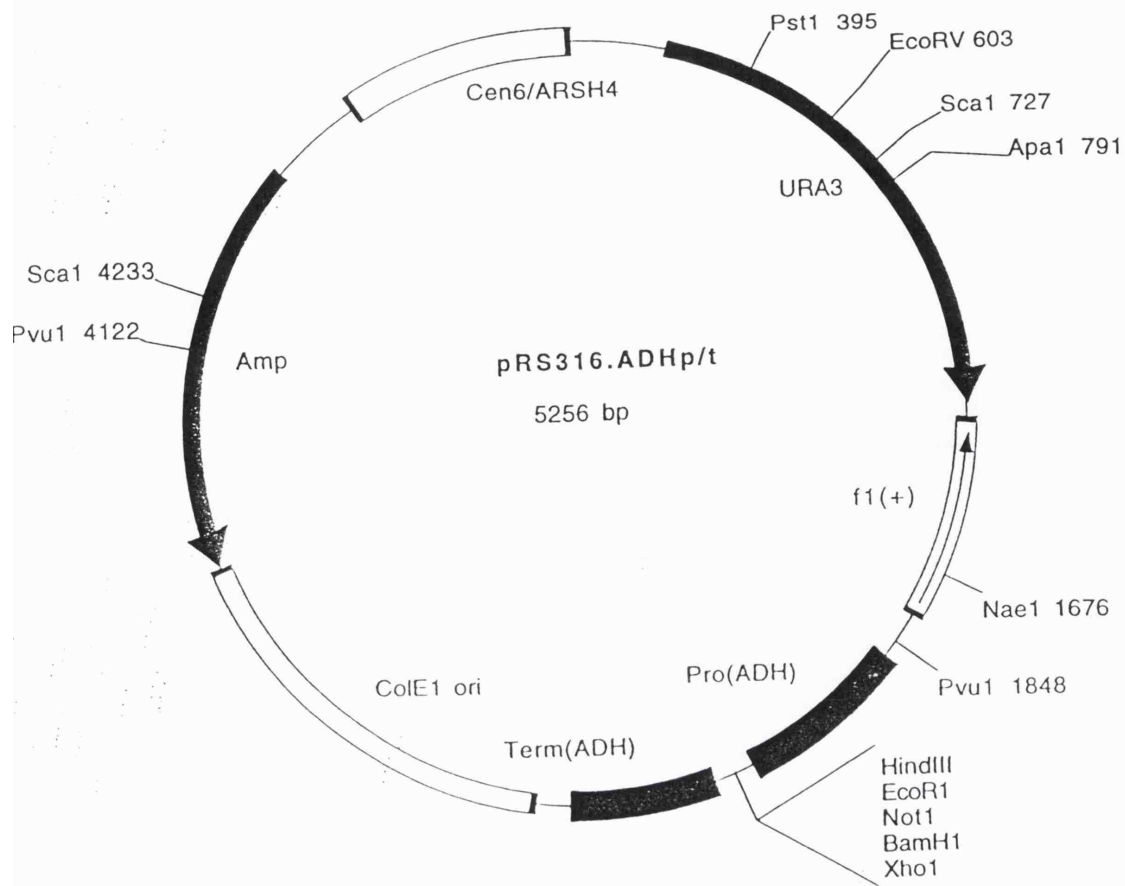


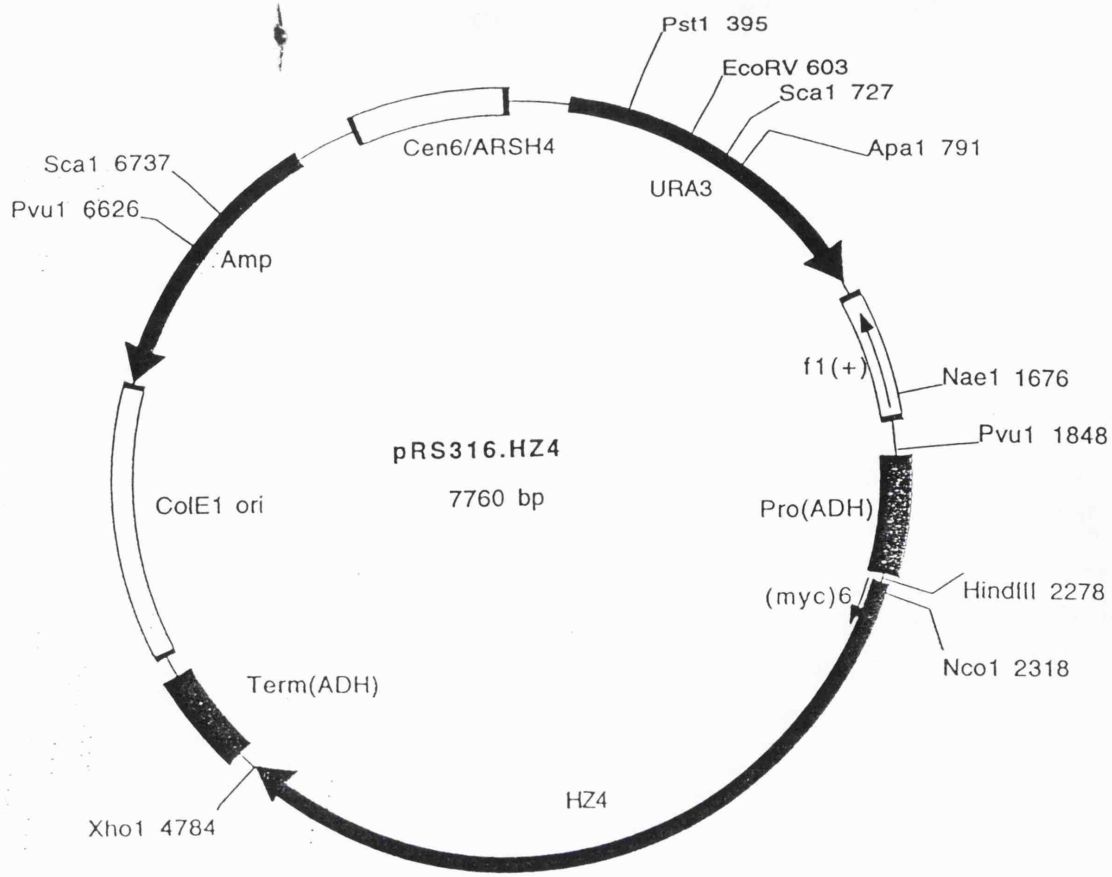


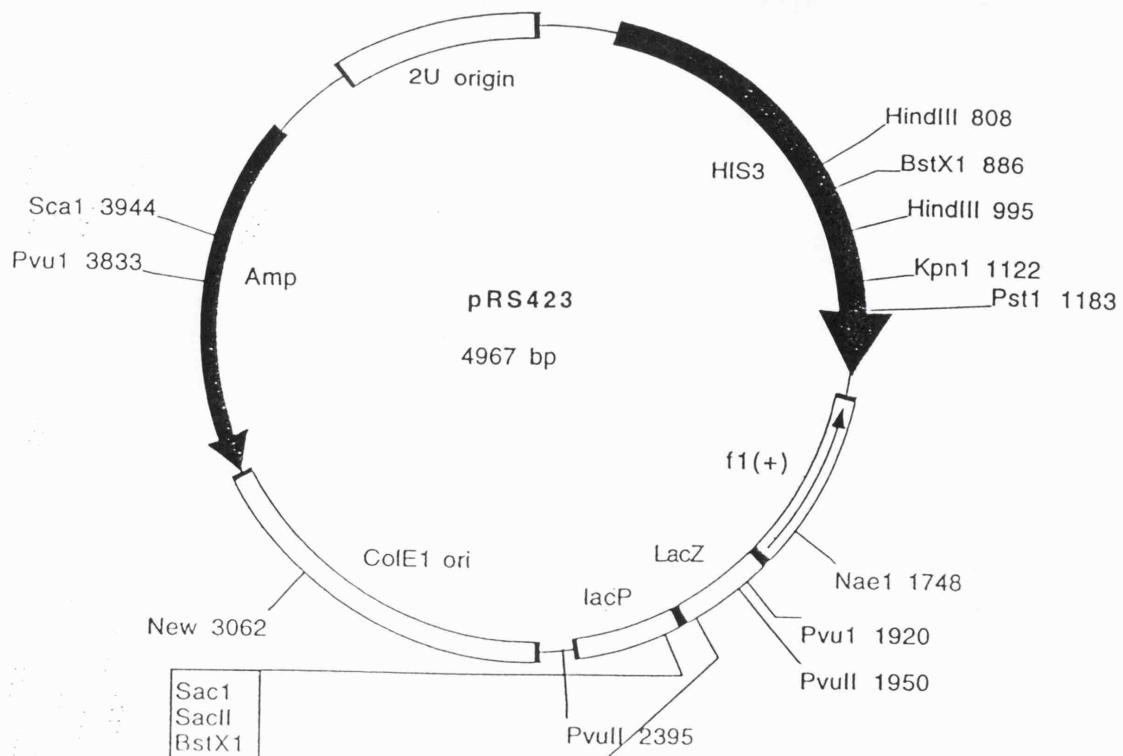
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SacI, SacII, BstXI, NotI, EagI, XbaI, SpeI, BamHI, SmaI, PstI, EcoRI, EcoRV, HindIII, ClaI, SalI, XhoI, ApaI, KpnI







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