Structure and Expression of the Human Serum Amyloid A Gene Family.

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Abstract.

Serum amyloid A (SAA) proteins comprise a family of phylogenetically conserved major acute phase reactants. To delineate the structure of the human SAA genes extensive Southern analysis was carried out using SAA probes. Multiple hybridizing fragments were detected with a range of restriction enzymes. Restriction fragment length polymorphisms (RFLPs) were identified for BglI, HindIII, NcoI and PstI which were inherited in a Mendelian fashion for the former two enzymes. Three contiguous clones isolated from a human λEMBL3 genomic library spanned 30kb, and contained a single SAA gene predicted to encode apoSAA1ß. *SAA1ß* was approximately 4kb long with 4 exons and showed >90% sequence homology to the previously characterized *SAA2ß* in exon, intron and 5'-controlling regions. A fourth λ clone encoded an allele of *SAA2ß*, namely *SAA2α* with HindIII, NcoI and PstI polymorphic sites. A third novel SAA gene (*SAA4*) was isolated from a cos202 cosmid library and mapped 10kb downstream of *SAA2*. These studies demonstrated that the human SAA gene family comprises four discrete loci.

The effects of inflammatory mediators on gene expression were studied following transient transfection of *SAACAT* reporter genes into HepG2 cells. Similar responses were obtained for *SAA1* and *SAA2* constructs following treatment with IL1ß, IL6 and TNFα. Whereas IL1 acts via the NFκB site in the SAA promoter, IL6 was shown to require both an intact NFκB site and a putative IL6 responsive element (IL6RE) upstream for full induction. *In vitro* bandshift and interference footprinting studies demonstrated binding of novel nuclear factors from IL6 treated HepG2 cells to both the NFκB region and an NFIL6-like recognition sequence at the IL6RE. IL1 and IL6 activated *SAACAT* synergistically. Functional studies indicated that this synergism was mediated through the NFκB site and in part through the putative IL6RE.

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List of Abbreviations.

Commonly used biochemical notation for molecular mass, nucleotides, amino acids *etc.* used throughout the text follow the guidelines outlined in *J. Biol. Chem.* (1990) **265**, 599-605. Other abbreviations used are listed below.

General.

α_1ACH	α_1-antichymotrypsin
α_1AGP	α_1-acid glycoprotein
α_1AT	α_1-anitrypsin
α_1I3	α_1-inhibitor III
α_1MAP	α_1-major acute phase protein
α_2M	α_2-macroglobulin
AA	Amyloid A
AMPS	Ammonium peroxodisulphate
APP	Acute phase protein
APR	Acute phase response
Bf	Complement factor B
C1INH	C1 inhibitor
C3, C4	Complement components
CAT	Chloramphenicol acetyl transferase
CM	Conditioned medium
Cp	Caeruloplasmin
CRP	C-reactive protein
DG	Diacylglycerol
FBN	Fibrinogen
FMF	Familial Mediterranean fever
FP	Female protein
(GM)CSF	(Granulocyte-macrophage) colony stimulating factor
HDL	High density lipoprotein
Hepes	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
hGH	Human growth hormone
Hp	Haptoglobin
HPBL	Human peripheral blood lymphocytes
HSF	Hepatocyte stimulating factor
Hx	Haemopexin
i.p.	Intraperitoneal
IFN	Interferon
IL1, 2 *etc*	Interleukin-1, 2 *etc*

IL1ra	IL1 receptor antagonist
IPTG	Isopropylthio-ß-D-galactoside
LBP	LPS-binding protein
LINE	Long interspersed nuclear element
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MoCM	Monocyte conditioned medium
MT	Metallothionein
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PEG	Polyethyleneglycol
PFGE	Pulsed field gel electrophoresis
PIC	Polymorphism information content
PKA	cAMP-dependent protein kinase
PKC	Calcium/phospholipid-dependent protein kinase
PMA	Phorbol-12-myristate-13-acetate
SAA	Serum amyloid A
SAP	Serum amyloid P component
SDS	Sodium dodecyl sulphate
SINE	Short interspersed nuclear element
ßME	ß-mercaptoethanol
TEMED	N,N,N'N'-tetramethylethylenediamine
TGF	Transforming growth factor
TNF	Tumour necrosis factor
Tris	Tris-(hydroxymethyl)-methylamine
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

Cell lines.

70Z/3	Murine pre-B cell
COLO16	Human squamous carcinoma
EL4	Murine thymoma
FAO	Rat hepatoma
H-35	Rat hepatoma
HeLa	Human cervical carcinoma
Hep3B	Human hepatoma
HepG2	Human hepatoma
HTC	Rat hepatoma
Jurkat	Human T leukaemic
NPLC/PRF/5	Human hepatoma
YT	Natural killer

Nuclear Factors and Related.

AP-1, 2	Activator protein 1, 2 *etc*
C/EBP	CCAAT/enhancer binding protein
CRE(B)	cAMP responsive element (binding protein)
HLH	Helix-loop-helix motif
HNF-1, 2	Hepatocyte nuclear factor 1, 2 *etc*
IκB	Cytoplasmic inhibitor of NFκB
NFκB	Nuclear factor binding κB motif
Oct1, 2	Octamer site binding proteins
SRF	Serum respose factor
v-*rel*	Avian reticuloendotheliosis virus
ZIP	Leucine zipper motif

List of published material.

The following list of presentations resulted from work carried out for this thesis.

Betts JC, Woo P. (1989) Effects of IL1 and IL6 on the transcription of the gene for the acute phase serum amyloid A (SAA) protein. *Br. Soc. Immunol.* Autumn meeting (Abstracts).

Betts JC, Edbrooke MR, Cheshire JK, Woo P. (1990) Induction of the human acute phase serum amyloid A genes by IL1 and IL6. (Abstract) *J. Cell. Biochem.* **14E**, 171.

Betts JC, Woo P. (1990) Characterization of the human SAA gene family. *VIth International Symp. on Amyloidosis* (Abstracts) p.76.

Betts JC, Woo P. (1990) Distinct control elements are required for IL1 and IL6 induction of SAA expression. *VIth International Symp. on Amyloidosis* (Abstracts) p.74.

Woo P, Betts JC, Edbrooke MR. (1991) The human serum amyloid A genes and their regulation by cytokines. In "Amyloid and Amyloidosis 1990", (Natvig JB, *et al*, eds.) Kluwer Academic Publishers, Dordrecht. pp. 13-19.

Betts JC, Edbrooke MR, Thakker RV, Woo P. (1991) The human acute phase serum amyloid A gene family: structure, evolution and expression in hepatoma cells. *Scand. J. Immunol.* In press.

Betts JC, Woo P. (1991) Involvement of NFκB-like and C/EBP-like factors in the acute phase induction of serum amyloid A gene transcription. *IRBM Symp. on Gene Expression During Liver Differentiation and Disease* (Abstracts) p.44.

Betts JC, Woo P. (1991) Interaction of IL1 and IL6 induced factors in the synergistic induction of SAA gene transcription. Manuscript in preparation.

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CHAPTER 1.

Introduction.

1.1 Inflammation and the Acute Phase Response.

1.1.1 Preface.

As a result of tissue injury or infection the defences of the body are stimulated to produce local inflammation, limiting the insult, and if the stimulus is great enough to mediate the acute phase response (APR).

The APR is characterized by marked alterations in the levels of a number of defined plasma proteins, as a result of injury. In this chapter the structure and regulation of these proteins will be assessed, the results of my studies on the human acute phase serum amyloid A (SAA) gene family will be presented and discussed in the ensuing chapters.

1.1.2 The Inflammatory Response.

The cardinal signs of inflammation are rubor, calor, turgor and dolor, resulting from changes in vessel tone and permeability. Soluble mediators released from blood leukocytes have been shown to cause changes in a number of cell types *in vitro*, which may lead to inflammation and activation. A number of these mediators, including interleukin 1 (IL1), interleukin 6 (IL6) and tumour necrosis factor (TNF) are responsible for acute phase protein (APP) induction.

Cell activation leads to the generation of biologically active mediators by several enzyme cascades including complement (see Kaplan and Siverberg, 1988), coagulation (Mann *et al*, 1988) and arachidonic acid metabolism (Irvine, 1982). These products can cause chemotaxis and activation of circulating blood cells (*i.e.* C5a, leukotriene B4), activation and modulation of endothelial cell function (bradykinin, thrombin) and non-specific cell lysis (complement proteins). Activated cells at the inflammatory site further enhance the response causing the release of platelet products, leukocyte proteases and free radical generation. Cytokines link this tissue activation stage and the systemic changes involving fever, haematopoiesis and the acute phase response potentiating and regulating the inflammatory response

1.1.3 The Acute Phase Response.

In 1930 Tillet and Francis when testing for antibody reactions found that sera from patients with acute infectious disease formed a precipitate with a solution of somatic C polysaccharide of pneumococcus. However this reaction differed from known antigen-antibody reactions in that it was calcium dependent and the titre was maximal during the active stage of the disease but decreased thereafter. The protein responsible for this phenomenon (C-reactive protein, CRP) was investigated by McCarty (1947) and an homologous protein found in other mammals (Anderson and McCarty, 1951; Gotschlich and Stetson, 1960). Initially CRP was only detected at the site of inflammation (Kushner and Kaplan, 1961) but later more sensitive methods implicated the liver as the site of

production (Hurliman *et al*, 1966). Thus a paradigm was created for the APPs: proteins whose liver production was modulated in response to inflammation.

In 1982 Kushner raised two questions about acute phase proteins:

i) what are the mechanisms by which inflammatory stimuli lead to increased biosynthesis of these proteins?

ii) what are the functional roles these proteins play in helping to survive acute insult and in the processes of inflammation and tissue repair? (Kushner, 1982). Rapid progress has been made in answering the first question with the characterization and cloning of a number of cytokines, however the roles of several major APPs are not conclusively defined in man. The current knowledge of APP structure function, and induction will be discussed in sections 1.1.4-1.1.6. Further information on the acute phase response can also be found in the reviews of Kushner, 1988; Schreiber, 1987; Fey and Fuller, 1989; Baumann, 1989; Fey and Gauldie, 1990; Sipe, 1990; Stadnyk and Gauldie, 1991.

1.1.4 Acute Phase Proteins: Classification and Measurement *In Vivo* and *In Vitro.*

The amplitude of induction and the actual proteins induced during the acute phase varies between species although the individual protein structures (and by inference function) are generally conserved. For example in man plasma concentrations of CRP and serum amyloid A (SAA) maybe elevated by several hundred fold above normal whereas serum amyloid P component (SAP) remains unchanged. In mice SAA and SAP increase dramatically whereas CRP changes little. Rats show yet another pattern of induction with the major APPs being α_2-macroglobulin (α_2M), whilst the concentration of CRP in normal plasma is high and alters only slightly during the APR. Kushner originally proposed that for a protein to be classified as an APP its concentration must alter by at least 25% during the APR (Kushner, 1982), although small changes maybe difficult to measure in plasma (Baumann, 1989) the development of *in vitro* models has largely alleviated this problem. Positive APPs are subdivided according to the magnitude of the change in their concentrations (see table 1.1) as well as according to their response to individual cytokines (table 1.2).

Table 1.1 Classification of Human APPs According to Alteration in Plasma Concentration During the APR.

Class	Fold Change	Protein
Group I	<2 fold	C3, C4, Bf
Group II	2-10 fold	ACH, AGP, AT, Cp, FBN, Hp, Hx
Group III	10->100 fold	CRP, SAA, LBP
Negative	Decrease	Albumin, transthyretin, ApoAI

See list of abbreviations for definitions.

	CRP	SAA	SAP (m)	α_2M (r)	ACH	MAP (r)	AGP (r)	Bf	C4	C3	FBN	Hp	Cp	Hx
In vivo Induction	10^2-10^3	10^2-10^3	10	>200	2-5	20	100	1.5	1.5	1.5	2	2-5	6-10	2
CM	+	+	+	+	+	+	+							+
IL1	+	+	+		+	-	+	+	-	+	-		+	+
IL6	+	+		+	+	+	+			+	+	+		
TNFα		+			+	-	+	+	-	+	-		+	+
IFNγ		+						+	+					
HSFI													+	
HSFII													x	+
HSFIII													x	
Refs	1	2	3	4	5	6	7	8	9	10	11	12	13	14

Table 1.2 **Modulation of Acute Phase Proteins by Inflammatory Mediators.**
The top row shows the maximal level of induction of human, mouse (m) and rat (r) APPs followed by the effects of cytokines in various systems. CM = monocyte conditioned medium; HSF = hepatocyte stimulating factor; + = stimulatory; - = inhibits induction by other cytokines; x = not induced; blank = data not available. Stimulation of rat APPs requires the presence of dexamethasone. 1) deBeer *et al.* (1982b); Goldman and Liu (1987); Ganter *et al.* (1989); 2) McAdam *et al.* (1978) and studies carried out in this laboratory; 3) Mortensen *et al.* (1983); Le and Mortensen (1986); Zahedi and Whitehead (1989); 4) Schreiber (1987); IL6 stimulation was achieved following i.p. injection of rhIL6 (Gehring *et al.*, 1987); CM produced a 54-fold increase in primary rat hepatocytes (Andus *et al.*, 1988); <2-fold increases occur in humans (Magielska-Zero *et al.*, 1988); 5) Baumann *et al.* (1984); Perlmutter *et al.* (1986a); Ganapathi *et al.* (1988b); May *et al.* (1988); Zuraw and Lotz (1990); 6) Baumann and Muller-Eberhard (1987); Magielska-Zero *et al.* (1987); Andus *et al.* (1988b); Bereta *et al.* (1989); 7) Andus *et al.* (1988a) ; CM induction was shown in the human hepatoma line Hep3B (Darlington *et al.*, 1986); 8) Perlmutter *et al.* (1986a, 1986b, 1986c); Nonaka *et al.* (1989); 9) Miura *et al.* (1987); 10) Darlington *et al.* (1986); Baumann *et al* (1987b); May *et al.* (1988); Mortensen *et al.* (1988); 11) Andus *et al.* (1988a); Castell *et al.* (1989); Huber *et al.* (1990); 12) Oliviero and Cortese (1989); 13) responses measured in Hep3B cells (Mackiewicz *et al.*, 1987); 14) Baumann and Muller-Eberhard (1987); Baumann *et al.* (1987a); Gauldie *et al.* (1987) Poli and Cortese (1989). HSFI and HSFII data for ACH, MAP, C3, FBN, Hp and Hx from H-35 cells (Baumann *et al.*, 1987a)

The problem of classification has been compounded by the diverse systems which have been used to measure both the induction and the steady state levels of APPs. Variable results are obtained dependent on the organism studied and whether the response is induced *in vivo* or *in vitro*. A wide range of induction levels have been found for SAA according to measurement of plasma levels (McAdam *et al*, 1978), production from primary hepatocyte cultures (Moshage *et al*, 1988), hepatoma cell lines (Ganapathi *et al*, 1988a, 1988b) or SV40 transformed hepatocytes (Liao *et al*, 1989). These discrepancies may be due to differing basal levels of proteins *in vivo* and *in vitro* and the dedifferentiation of both primary hepatocytes and transformed cell lines which occurs in culture. Primary hepatocyte cultures undergo trauma on isolation and maybe contaminated with other cell types such as endothelial cells and Kupffer cells which produce cytokines and thus alter hepatocyte function (Guillouzo *et al*, 1984). Indeed Koj *et al* (1984) noted that hepatocytes isolated from rats synthesized proteins *in vitro* at a rate more consistent with those seen *in vivo* during the APR.

APPs have been measured by two-dimensional gel electrophoresis, or crossed immunoelectrophoresis using antibodies to total serum (Baumann *et al*, 1984). The availability of polyclonal and monoclonal antibodies has allowed measurement by ELISA (Goldman and Liu, 1987), rocket electrophoresis (Marinkovic and Baumann, 1990), Western blotting (Dente *et al*, 1988), radioimmunoassay (Darlington *et al*, 1986) and immunoprecipitation of labelled proteins (Andus *et al*, 1988b). As the genes for the majority of the APPs have been cloned mRNA levels and transcriptional responses have been quantitated. The establishment of cell lines carrying AP genes in high copy number allows simple measurement of responses to individual stimuli, particularly for those proteins with basal levels close to the limits of detection of most assays (*e.g.* Woo *et al*, 1987).

Elegant studies *in vivo* in several species have charted the increases in various expression parameters of individual APPs. For example Syrian hamster caeruloplasmin (Cp) showed peaks in transcription, mRNA and protein levels 18, 24 and 120 hours respectively following turpentine injection (Gitlin, 1988), whereas murine serum amyloid P (SAP) showed only a 28 hour delay between peak mRNA and peak plasma protein (Zahedi and Whitehead, 1990).

1.1.5 Structure and Function of Acute Phase Proteins.

1.1.5.1 Pentraxins.

CRP is the prototypical APP and one of the major APPs in humans (see section 1.2 for discussion of the other major APP, SAA). CRP together with SAP and hamster female protein (FP) belong to the pentraxin family. Members of this family are comprised of 5 identical subunits non-covalently linked to form a discoidal structure (Pepys and Baltz, 1983). In man resting levels of CRP ($<1 mgml^{-1}$) increase to $200 mgml^{-1}$ within a few hours of insult (*e.g.* myocardial infarction; deBeer *et al*, 1982b). CRP is encoded by a single locus (Le *et al*, 1985; Woo *et al*, 1985) and is phylogenetically conserved.

Molecules with calcium-dependent C-polysaccahride binding have been isolated from diverse species including the invertebrate *Limulus polyphemus* (Robey and Liu, 1982), thus APPs may represent a response to insult which developed early in evolution. However a clear functional role has not been elucidated for CRP. The various ligands to which CRP can bind suggest that it may be involved in the clearance of "abnormal" constituents from the serum during inflammation (Pepys and Baltz, 1983). Activation of complement and effects on cells activated during inflammation have also been demonstrated (see table 1.3).

A central concern of studies on the APR has been to elucidate the mechanisms by which the major APPs undergo both a rapid and large increase in production. Theoretically proteins could be released from large intracellular stores or be products of *de novo* protein synthesis. In fact the latter appears to be the case, a process which can be broken down into several potential levels of control; involving increases in transcription, mRNA stability, translation and secretion rate. Alterations in each of these parameters occurs during CRP induction (see table 1.4). Attempts to identify the cytokine(s) responsible for such changes have produced variable results. Moshage *et al* (1988) found that IL1, IL6 and TNF could individually upregulate CRP in primary human hepatocytes whereas only IL6 was effective in the study of Castell *et al* (1989). In hepatoma lines only IL6 alone stimulated the endogenous CRP gene in NPLC/PRF/5 but in Hep3B cells no cytokine was effective alone (Ganapathi *et al*, 1988a; 1988b; Goldman and Liu, 1987). However when the human CRP promoter was transfected into Hep3B cells induction occurred with IL6 and combinations of IL1 and IL6 were synergistic (Ganter *et al*, 1989).

SAP is a normal plasma constituent in humans and is also found in glomerular basement membrane, alveolar capillary walls and in intercellular structures in cardiac and smooth muscle (Dyck *et al*, 1980). This protein is found universally associated with amyloid deposits regardless of the nature of the fibrillar component (see section 1.2). Despite homology to CRP, SAP does not bind phosphorylcholine or C-polysaccharide and was isolated through calcium dependent binding to agarose columns (Pepys *et al*, 1977). Like CRP, SAP maybe involved in clearance of debris released by lysed cells as high affinity binding to chromatin has been demonstrated (Pepys and Butler, 1987).

In mice SAP levels peak at 6-20 times basal levels 24-36 hours after an inflammatory stimulus (dependent on strain and stimulus) returning to preinflammatory levels by 72 hours (Mortensen *et al*, 1983; Zahedi and Whitehead, 1989). *In vitro* monocyte conditioned medium (CM) caused a 7 fold increase in the number of hepatocytes synthesizing SAP, although IL1 alone increases SAP synthesis other cytokines are responsible for increasing the number of hepatocytes (Le and Mortensen, 1986). Mouse SAP shares 70% amino acid homology to human SAP and the genes are homologous in the 5'-flanking region between -117 and -41 (Dowton *et al*, 1985; Whitehead and Rits, 1989). This homology is notable in respect of the divergent regulation of SAP in the 2 species. Analysis of rat liver mRNA during inflammation

revealed a small (<2 fold) increase in SAP peaking at 24 hours after stimulation (Dowton and McGrew, 1990).

A further member of the pentraxin family, which has been designated Syrian hamster female protein (FP), circulates as a single pentameric disc and binds phosphorylcholine and agarose in a calcium-dependent manner (Coe, 1977; Dowton *et al*, 1985). FP is regulated in the Syrian hamster under AP conditions and by sex steroids. Resting levels are 100 to 1000 times higher in females than in males but whereas 5 to 10 fold increases occur in males during the APR a 50% drop occurs in females (Coe and Ross, 1983). However in the closely related Armenian hamster FP levels increase during the APR irrespective of gender (Dowton and Waggoner, 1989).

1.1.5.2 Proteinase Inhibitors.

APPs are diverse in nature and include several proteinase inhibitors two of which, α_2M and α_1-major acute phase protein (α_1MAP) are major APPs in the rat. α_2M is a broad range proteinase inhibitor comprised of four 185kDa polypeptide subunits encoded by a 36 exon gene spanning 50kb (Hattori *et al*, 1989b). In addition to its proteinase inhibiting properties α_2M binds a wide range of molecules including endopeptidases, mitogens, LPS, cytokines and ions. Binding to cytokines may promote or inhibit their activities, it has been suggested that α_2M slowly inactivates IL2 but protects IL6 (reviewed by James, 1990). This molecule may therefore be important in modulating the effects of various inflammatory mediators. The reason why α_2M is not an APP in other species is not clear, however as a major plasma protein in humans it would be available to carry out these functions without upregulation.

α_2M is increased 320-fold in a corticosteroid-dependent fashion in the rat following experimental inflammation (Schreiber *et al*, 1982). Although not regarded as an APP in humans variations between 2 and 4mgml^{-1} may occur according to age and sex and small increases in secretion from HepG2 cells in culture (<2 fold) have been measured in response to 48 hours stimulation with IFNγ (Magielska-Zero *et al*, 1988). Acute phase induction of rat α_2M could only be partially reproduced by intraperitoneal injection of rhIL6 giving a 20 fold increase in mRNA and was male-specific in Wistar rats (Geiger *et al*, 1988). Even lower responses were generally found in rat primary hepatocyte cultures: 3 fold in response to IL6 and 5 fold with CM (Koj *et al*, 1984; Bereta *et al*, 1989), although a 54 fold increase in secreted protein and a 2-3 fold increase in mRNA was detected in one study (Andus *et al*, 1988b).

Other APPs include members of the serpin superfamily such as α_1-antitrypsin (AT, also known as α_1 proteinase inhibitor), C1 inhibitor (C1INH), α_1-antichymotrypsin (ACH) and angiotensinogen (the latter is not a proteinase inhibitor but is structurally related to the serpins). The major physiological role of AT is believed to be protection of alveolar connective tissue against attack by neutrophil elastase (Travis and Salvesen, 1983). A 2-4 fold increase of AT during the APR helps to inhibit elastase which diffuses

Table 1.3 Known and Putative Functions of Acute Phase Proteins.

CRP	Clearance of abnormal plasma constituents	1
	Opsonization	2
	Modulation of inflammatory cell function	3
SAA	Immunosuppressive	4
	Altered HDL metabolism	
	Collagenase induction	
SAP	Cell attachment	5
	Clearance of abnormal plasma constituents	6
	Elastase inhibition	7
α_2M	Proteinase inhibitor	8
	Modulation of cytokine activity	9
α_1AT	Elastase inhibition	10
ACH	Proteinase inhibition	
MAP	Cysteine proteinase inhibition	11
	Source of inflammatory peptides	12
Hx	Scavenges haem	
Hp	Scavenges Hb	13
	Cathepsin inhibition	
Cp	Scavenger of superoxide anion	14
LBP	Opsonin	15
MT	Sequesters zinc	16
	Binds radicals	
AGP	Immunosuppressive	17

Data which support the roles shown for the proteins can be found in the following references: 1) Tillet and Francis (1930); Narkates and Volanakis (1982); Robey *et al* (1985); Duclos *et al* (1988); DuClos (1989); 2) Pepys and Baltz (1983); 3) Buchta *et al* (1987); Robey *et al* (1987); Ballou *et al* (1989); 4) see section 1.2; 5) Dhawan *et al* (1990); 6) Pepys and Butler (1987); 7) Li and McAdam (1984); 8) Schreiber (1987); 9) James (1990); 10) Travis and Salvesen (1983); 11) Esnard and Gauthier (1983); 12) Cole *et al* (1985); 13) Engler (1982); 14) Goldstein *et al* (1979); 15) Schumann *et al* (1990); 16) Schroeder and Cousins (1990); 17) Friedman (1983); Cheresh *et al* (1984).

away from the site of inflammation. C1INH is produced by diverse cell types and is the major plasma inhibitor of C1, kallikrein and Hageman factor. C1INH belongs to the group of proteins inducble by IL1 with secretion from human hepatocyte and hepatoma lines peaking at 10 fold in response to IFNγ after 4 days (Zuraw and Lotz, 1990). ACH is a well characterized APP which inhibits chymotrypsin, cathepsin G and chymase (reviewed by Travis and Salvesen, 1983). ACH levels which increase 2-5 fold *in vivo* are elevated in response to monocyte and COLO16 CM in HepG2 and Hep3B cells (Baumann *et al*, 1984; Ganapathi *et al*, 1988b) as well as IL1, TNF or IL6 in the same cells (Perlmutter *et al*, 1986a; Ganapathi *et al*, 1988b; May *et al*, 1988; Zuraw and Lotz,

1990). Primary human hepatocytes cultured in dexamethasone increased ACH production with rhIL6 (Castell *et al*, 1989).

The major acute phase α_1-protein (MAP) of the rat (also known as thiostatin, T-kininogen and immunochemically identical to α_1-cysteine proteinase inhibitor, CPI) becomes the most abundant protein in the blood during the APR. IL6 is the major inducer of MAP in primary hepatocytes and hepatoma cells whereas IL1 and TNF are ineffective (Baumann and Muller-Eberhard, 1987; Magielska-Zero *et al*, 1987; Bereta *et al*, 1989; Andus *et al*, 1988b). Injection of rhIL6 also induced MAP 10 fold in rats (Geiger *et al*, 1988).

1.1.5.3 Acute Phase Proteins with Transport/Binding Function.

An important role for a number of APPs is the conservation of tissue/cellular components and the neutralization of harmful products released as a result of tissue injury. For example, haemopexin (Hx) scavenges haem released by *in vivo* haemolysis, and is endocytosed by the liver where the haem is processed further. A 6 fold increase in Hx mRNA was found in human hepatoma cells following treatment with IL6 (Poli and Cortese, 1989). Haptoglobin (Hp), an $(\alpha ß)_2$ tetrachain glycoprotein fulfils a similar function binding free haemoglobin. Hp may also function as an inhibitor of cathepsins B and L (Engler, 1982) and is descended from the serine proteases (Doolittle, 1980). Although classified as a minor APP in humans a 28 fold increase was achieved with IL6 when the Hp promoter was transfected into Hep3B cells (Oliviero and Cortese, 1989). The higher levels of induction of Hx and Hp *in vitro* may reflect a rapid plasma turnover following substrate binding particularly if haemolysis is widespread.

Caeruloplasmin (Cp) carries >95% of plasma copper and is an effective scavenger of superoxide anion, thus may reduce the toxicity of free radicals released during the oxidative response of neutrophils (Goldstein *et al*, 1979). Hepatic Cp levels increase 6-10 fold within 24 hours af an inflammatory stimulus in human, rat and hamster. In addition to increased hepatic expression, mRNA was detected in the choroid plexus, yolk sac, placenta and testis in rats (Aldred *et al*, 1987). Extrahepatic expression has also been found for a number of other APP genes and may reflect requirement for these products at localized sites at different stages of inflammation and/or development.

A protein recently identified as an APP is LPS binding protein (LBP). Steady state levels of LBP <0.5ugml^{-1} increase to 50ugml^{-1} 24 hours after AP induction (Tobias *et al*, 1985, 1986), thus LBP is implicated as a major APP in man. This 60kD protein, synthesized in the liver, has a binding site for the lipid A moiety of LPS. LBP can opsonize particles displaying LPS as well as intact Gram negative bacteria, mediating attachment to macrophages via a specific cellular receptor, CD14, a monocyte differentiation antigen (Schumann *et al*, 1990 Wright *et al*, 1990). Thus LBP provides a first line defence against invading particles and by binding CD14 as a LBP-LPS complex triggers the release of TNFα which can further modulate the APR.

1.1.5.4 Other Acute Phase Proteins.

A number of APPs do not readily fall into the functional/ structural classes defined above and include α_1-acid glycoprotein (AGP), a major constituent of normal plasma which is universally elevated during the AP in human, mouse and rat. However the magnitude of the increase in humans is small (10 fold) compared to the rat (100 fold). There are at least 2 AGP genes in humans and both human and rat genes have 6 exons and span 4kb (Dente *et al*, 1985; Reinke and Feigelson, 1985). Transcriptional studies revealed a proximal responsive element (PRE) in the rat gene which mediated dexamethasone dependent expression (Baumann and Maquat, 1986) whereas an upstream region (distal responsive element or DRE) was necessary for IL1, IL6, TNFα and LIF modulation (Prowse and Baumann, 1988; Won and Baumann, 1990). Studies on AGP *in vitro* have shed some light on the question as to whether glucocorticoids are a requirement for human AP induction as in rats. In HepG2 cells AGP did not respond to dexamethasone alone but it could augment the induction by IL1, IL6 and HSFII (Prowse

Table 1.4 Alteration in Expression Parameters of APPs During Induction.

Protein	Level of Control			Other
	Transcription	mRNA stability	Secretion	
1) CRP	+	+	+	Glycosylation
2) SAA	++	+		
3) SAP	+			
4) α_2M	+	++		
5) MAP	+			
6) Hx	+			
7) Hp	+			
8) Cp	+			
9) AGP	+	+		Glycosylation
10) FBN	+			

1) IL6 increases transcription whereas IL1 stabilizes mRNA in transfected Hep3B cells (Ganter *et al*, 1989); an increased secretion rate was found in rabbit hepatocytes (Macintyre *et al*, 1985) but not in human hepatoma cells (Hu *et al*, 1988); 2) Increased transcription in human cells (studies in this lab.); increased transcription and mRNA stabilization in mouse (Lowell *et al*, 1986b; Rienhoff, 1989); increased poly(A) tail length in mouse and dog (Brissette *et al*, 1989; Sellar *et al*, 1991); 3) Le and Mortensen (1986); Zahedi and Whitehead (1989); 4) 214 fold increase in mRNA with 3 fold increase in transcription in rat (Gehring *et al*, 1987); 5) Birch and Schreiber (1986); differential usage of 3 transcription initiation sites of the MAPI gene in inflammation (Kageyama *et al*, 1987); 6) Poli and Cortese (1989); 7) Morrone *et al* (1988); Oliviero and Cortese (1989); 8) Measured in Syrian hamster (Gitlin, 1988); 9) mRNA stabilization occurs in the rat (Vannice *et al*, 1984; Reinke and Feigelson, 1986; Birch and Schreiber, 1986) with an increase in poly(A) tail length (Shiels *et al*, 1987); increased glycosylation (Conner *et al*, 1990; van Dijk *et al*, 1991); 10) Birch and Schreiber (1986).

and Baumann, 1988). However when the rat AGP gene was transfected into HepG2 cells induction could be achieved by dexamethasone alone indicating that in this case the differing glucocorticoid responses were due to information carried within the genes.

The complement system provides an important defence against bacterial infection. The concentrations of several of the complement proteins are increased in the plasma during inflammation. Complememt components C3, C4 and Bf fall into the group I class of APPs in that they increase by 50% during inflammation (Kushner, 1988) although larger increases have been recorded *in vitro* (Darlington *et al*, 1986; Perlmutter *et al*, 1986a). The various complement proteins show differential responses during the APR. For example the structurally and functionally homologous Bf and C2 genes are closely linked in the MHC class III region, the 3' end of C2 being 450bp from the 5' end of Bf. However C2 is not an APR and is not responsive to the Bf-inducing cytokines IL1 and TNF (Perlmutter *et al*, 1986a, 1986b). The C4 genes are also closely linked and homologous but have distinct complement activating functions. These genes are regulated by IFNγ but not IL1 or TNF (Miura *et al*, 1987). C3 synthesis is modulated by IL1, TNF and IL6 in a number of systems (Darlington *et al*, 1986; Baumann *et al*, 1987a; May *et al*, 1988; Mortensen *et al*, 1988).

Fibrinogen (FBN) is required wherever damage to blood vessels occurs and expression increases over 2 fold in man, mouse and rat. The formation of FBN Bß chains is the rate-limiting step in assembly as Aα and γ chains can be provided by intracellular pools (Yu *et al*, 1984) although mRNAs for all 3 chains increase under inflammatory conditions (Fuller *et al*, 1985). A potential feedback mechanism may operate during the APR as synthesis of HSF by macrophages was shown to be controlled by plasmin derived fragments of fibrin or FBN (Ritchie *et al*, 1982). Although IL1 has been recorded as increasing FBN expression in a human hepatoma line (Zuraw and Lotz, 1990), IL6 appears to be the major regulator in humans and rats (Andus *et al*, 1988a; Castell *et al*, 1989; Huber *et al*, 1990).

1.1.6 Negative Acute Phase Proteins.

Whereas the most conspicious changes during the APR involve the increased plasma levels of several proteins, the concentration of a number of plasma proteins declines. Albumin production falls most significantly in humans and can drop to 40% of steady state levels. It has been proposed that in this way albumin may function as a metabolic adaptor (Schreiber, 1987), a reduction of biosynthesis in the liver may allow general enzymes and substrates to become available to synthesize the positive APPs. The significance of the reduction of other negative APPs such as transthyretin, apoAI and α_1I3 in the rat is less clear. α_1I3 is a broad range proteinase inhibitor like α_2M and the two have structurally similar genes (Braciak *et al*, 1988) however transcription of the α_1I3 gene drops 13 fold during inflammation (Abraham *et al*, 1990). Transferrin has been described as both a negative (Perlmutter *et al*, 1986a; Koj *et al*, 1984; Castell *et al*, 1989) and a positive APP (Schreiber, 1987). However increases in concentration are

delayed in comparison to other APPs by 2-3 days, it therefore appears likely that after an initial decrease, levels increase as a secondary response to decreased plasma iron.

1.2 Serum Amyloid A.

1.2.1 Background.

Amyloid deposits occur in a number of disease states compromising organ function. Classically these deposits stain with Congo red, showing apple green birefringence under polarized light (Husby and Sletten, 1986). Amyloid is composed of a fibrillar component which can form ß-pleated sheet structures (Eanes and Glenner, 1968) associated with SAP and glycosaminoglycans. The fibrillar component was found to vary structurally in different diseases: whereas they were composed of fragments of immunoglobulin light chains in primary and myeloma associated amyloidosis (Glenner *et al*, 1971) and transthyretin in familial polyneuropathies (Costa *et al*, 1978), those associated with amyloidosis secondary to inflammatory diseases and the autosomal recessive disease familial Mediterranean fever (FMF) bore no relationship to known immunoglobulins (Benditt and Eriksen, 1971; Husby *et al*, 1972; Levin *et al*, 1972). The first primary sequences of this protein, variously called A-component, non-immunoglobulin acid soluble fraction (ASF) and amyloid of unknown origin (AOU) revealed a 76 amino acid molecule with microheterogeneity at residue 52 (Levin *et al*, 1972). Antiserum raised against the A-component was found to cross-react with a circulating protein in the serum of patients with chronic disease and to a lesser extent in healthy individuals (Levin *et al*, 1973; Husby and Natvig, 1974; Benson *et al*, 1975). Later measurements of normal and AP sera (Rosenthal and Franklin, 1975; Gorevic *et al*, 1976) and individuals injected with etiocholanolone (McAdam *et al*, 1978) demonstrated that amyloid A related serum component (SAA) was an APP. That SAA was the precursor to AA was supported by several lines of evidence, firstly they were immunologically related and shared N-terminal sequence identity; secondly monocyte derived proteases could degrade SAA to an AA-like fragment (Lavie *et al*, 1980; Skogen *et al*, 1980) and finally the accumulation of human AA in murine amyloid occurred following the introduction of human AP HDL into mice (Husebekk *et al*, 1985). SAA was found to circulate in the HDL_3 plasma fraction (Benditt and Eriksen, 1977) and isolation of SAA from this fraction demonstrated that up to six isoforms were present in a single individual (Bausserman *et al*, 1980; Marhaug and Husby, 1981).

1.2.2 SAA Function.

Analyses of the primary structure of SAA and AA proteins from species as diverse as man and the Pekin duck has demonstrated strong phylogenetic conservation, this fact together with the rapid inducibilty during inflammation is indicative of an important functional/protective role. The effects of SAA on different cell types has been

studied, for example immunosuppressive effects have been described: SAA isolated from CBA mice suppressed the *in vitro* antibody response to sheep red blood cells (Benson *et al*, 1975; Benson and Aldo-Benson, 1979; Peristeris *et al*, 1988), possibly via interference with macrophage/ T cell interactions (Aldo-Benson and Benson, 1982). In other studies SAA was shown to inhibit the inflammatory response of activated phagocytes by inhibiting the generation of toxic oxygen species (Greeley *et al*, 1987) as well as inhibiting thrombin-induced platelet aggregation (Zimlichman *et al*, 1988). Reports at a recent meeting also described the inhibition of PMA-induced differentiation of HL60 cells (Goldfarb *et al*, 1990). Thus SAA may modulate cellular function during the inflammatory response.

The properties of SAA in relation to its status as an apoprotein have also been considered. ApoSAA displaces apoAI from the HDL particle (Coetzee *et al*, 1986) and apoSAA turnover was found to be more rapid than that of apoAI, thus leading to the proposal that apoSAA may function to clear HDL more rapidly. In addition apoSAA was catabolized independently of other apoproteins in the liver (Bausserman *et al*, 1987; Gollaher and Bausserman, 1990) by a mechanism which was not saturable even at peak levels (Tape and Kisilevsky, 1990). Attempts to explain the physiological relevance of this have met with limited success, for example when LPS was mixed with rabbit AP serum the major complex formed was an LPS-HDL complex containing apoSAA, however no difference was found in the clearance of LPS in normal and AP rabbits (Tobias *et al*, 1982). Cholesterol metabolism appears to be influenced by apoSAA due to interference of the lecithin-cholesterol acyl transferase reaction which takes place on HDL thus reducing the formation of cholestryl ester during inflammation (Steinmetz *et al*, 1989).

Rabbit synovial fibroblasts treated with PMA were found to increase expression of a 14kD protein with amino acid homology to SAA. When purified from polyacrylamide gels this protein had the ability to induce collagenase synthesis in both rabbit and human fibroblasts (Brinckerhoff *et al*, 1989). Mitchell *et al* (1991) have cloned the corresponding cDNA from rabbit fibroblasts and have demonstrated the specificity of the response using antibodies against SAA and suggest that c-*jun* maybe a component of the pathway mediating collagenase induction. This cytokine-like effect of SAA may have important consequences in diseases such as rheumatoid arthritis when SAA levels are high.

1.2.3 SAA Genes and Their Regulation.

In the mouse SAA is encoded by a gene family comprising 3 expressed genes (*mSAA1-3*) and a pseudogene (Lowell *et al*, 1986a) which have been mapped to a 79kb region (Yamamoto *et al*, 1987) of mouse chromosome 7 (Taylor and Rowe, 1984).The *SAA1* and *SAA2* genes are highly conserved maintaining 96% sequence homology from 288bp 5' of exon 1 to 443bp 3' of exon 4, whereas *SAA3* has diverged substantially. Although *SAA1-3* contribute equally to a >1000-fold increase in mRNA levels in the liver

following LPS injection (Lowell *et al*, 1986b) only proteins encoded by *SAA1* and *SAA2* circulate in HDL during the APR. The significant time lapse between peak transcription (>300-fold at 3 hours) and peak mRNA levels (at 9-12 hours) indicate that both transcriptional and post-transcriptional processes are involved in the increase in SAA (Lowell *et al*, 1986b). More recent studies on the *SAA3* gene which is expressed in the mouse liver-derived cell line, BNL also suggest a post-transcriptional mechanism for induction by conditioned medium. Changes in transcription rates and mRNA stability were not detected following treatment although mRNA levels increased >100 times, thus attenuation of an mRNA degrading activity may be involved (Rienhoff and Groudine, 1988). A CM-responsive element between -185 and -118 and a potential liver-specific element between -118 and -63 have been identified in the *SAA3* gene (Huang *et al*, 1990).

The murine SAA genes show differential patterns of expression both temporally and spatially following various inflammatory stimuli. After LPS administration *SAA3* mRNA levels in the liver peak at 12 hours and decline slowly to basal levels by 48 hours, in contrast *SAA1* and *SAA2* levels peak at the same time but remain at this level until 36 hours when they drop sharply (Lowell *et al*, 1986b). When casein is used to induce inflammation *SAA3* mRNA levels in the liver are barely detectable whereas those for the other 2 genes increase dramatically (Meek and Benditt, 1986). Although hepatocytes are the major site of SAA expression, extrahepatic expression was also found in the kidney, spleen, heart, lung and intestine after LPS or IL1 treatment (Ramadori *et al*, 1985a). Three groups of extrahepatic sites were defined on the basis of the genes expressed therein after LPS treatment of mice: those where all three genes were expressed (liver, kidney); expression of *SAA1* and *SAA3* only (small and large intestine) and lastly predominantly *SAA3* expression (most tissues examined) (Meek and Benditt, 1986). Of particular note was the high level of *SAA3* induction in peritoneal macrophages which reached the same as that achieved in the liver (Meek and Benditt, 1986; Rokita *et al*, 1987). Extrahepatic *SAA3* expression was confined to adipocytes adherent to the adrenal glands and aorta, and within connective tissue adherent to the intestinal tract and within the lung. *SAA3* expressed in the spleen was associated with the mononuclear cells in the parafollicular zones (Benditt and Meek, 1989a). RNA was also detected in the epithelial cells of the proximal and distal convoluted tubules and ileum mucosal membranes (Meek *et al*, 1989). It will be interesting to see the role (if any) of SAA expressed at these sites. For a review on the structure and expression of the murine SAA genes see Rienhoff *et al* (1990).

Recent insights into SAA gene structure in other species has been obtained with evidence for multiple SAA genes. In the Syrian hamster a genomic clone containing a gene structurally homologous to the murine genes has been characterized as well as two distinct cDNA clones (Webb *et al*, 1989). Hybridization of Northern blots from various tissues with probes specific to the *ShSAA1/2* and *ShSAA3* genes showed differential expression of these genes which included extrahepatic sites (Webb *et al*, 1989). Two

mink cDNA clones have been obtained and the corresponding genes are expressed in the liver and to a lesser extent in the lung and brain during the APR (Marhaug *et al*, 1990). 5 distinct cDNAs have been isolated from a library of dog liver, consistent with the presence of 3 expressed gene loci (Sellar *et al*, 1991).

Rats are anomalous as unlike all the other species studied no SAA is found associated with the HDL during the APR (Baltz *et al*, 1987). However using the murine gene specific probes Meek and Benditt identified an *SAA1/2*-related mRNA, about 200 nucleotides shorter than the 600 nucleotide murine mRNA in rat AP liver. An *SAA3*-related mRNA of 600 nucleotides was found in the lung, ileum and large intestine but not in the liver after LPS injection. *In vitro* translation of the liver mRNA produced a protein of 8kD (*i.e.* 4kD smaller than expected for a full length SAA protein). The *SAA1/2*-related mRNAs did not hybridize to oligonucleotides specific to the phylogenetically conserved domain (Meek and Benditt, 1989b). Thus rats like other species appear to have a SAA gene family but mutation has occurred at the *SAA1/2* locus resulting in deletion of coding regions either in the genome or due to aberrant splicing. Multiple amino acid sequences for SAA in the Abyssinian cat (Kluve-Beckerman *et al*, 1989), microheterogeneities in the protein sequence of the horse (Sletten *et al*, 1989) and the presence of distinct electrophoretic forms in the rabbit (Tobias *et al*, 1982) are indicative of multiple genes in these species as well.

In terms of amyloid formation specific isotypes appear to be deposited in mouse and mink (SAA2) (Waalen *et al*, 1980; Hoffman *et al*, 1984; Meek *et al*, 1986; Shiroo *et al*, 1987). In the mouse this may be due to secondary structure differences between SAA1 and SAA2 proteins with SAA2 having about half the α-helical content as shown by circular dichroism studies and a greater tendency to aggregate than SAA1 (McCubbin *et al*, 1988). Certain mouse strains which are resistant to amyloid formation, such as SJL mice were found to be defective in SAA2 transcription (Yamamoto, 1986). However other factors are involved as amyloid-resistant A/J mice have high levels of SAA2 during inflammation (Hebert and Gervais, 1990). In humans more than one isotype is found in fibrils.

Studies on human SAA genes have resulted in the accumulation of numerous SAA/AA protein sequences, cDNA sequences and the complete sequence of two genomic clones. Six major isoforms have been isolated from human AP HDL (Baussermann *et al*, 1980; Marhaug and Husby, 1981). All individuals have one of three isoform patterns which can be equated to two gene loci (Strachan *et al*, 1985, 1989a). Each gene product has a smaller counterpart which lacks the N-terminal arginine (Strachan *et al*, 1989a). The first full apoSAA sequence identified a 104 amino acid protein with Val^{52} and Ala^{57} or *vice versa* (SAA1α and ß respectively; Parmelee *et al*, 1982). SAA2 was found to have 7 amino acid substitutions compared to SAA1 with either His (SAA2α) or Arg (SAA2ß) at residue 71 (Dwulet *et al*, 1988). cDNAs corresponding to SAA1α (pA1,; Sipe *et al*, 1985), SAA2α (pA10; Sipe *et al*, 1986; pSAA82; Kluve-Beckerman *et al*, 1986a; pAS_8;

Steinkasserer *et al*, 1990) and SAA2ß (Kluve-Beckerman *et al*, 1988; pAS_2, Steinkasserer *et al*, 1990) have been cloned from AP livers. Indeed all three forms have been cloned and their corresponding proteins isolated from a single individual (Kluve-Beckerman *et al*, 1988). A genomic clone λSAAg9 which encodes SAA2ß has a similar 4 exon structure to the murine genes (Woo *et al*, 1987) as does GSAA1 (*SAA3*) (Sack and Talbot, 1989). The predicted SAA3 protein sequence shows strong homology in the N-terminal region with the rabbit SAA collagenase inducer desribed above and thus is a candidate for a human homologue. The human genes are located on chromosome 11p and restriction fragment length polymorphisms (RFLPs) have been found in both normal individuals (Kluve-Beckerman *et al*, 1986b) and those with FMF (Sack, 1988) although no linkage has been found between the SAA locus and FMF (Shohat *et al*, 1990).

Expression studies carried out on the human SAA2ß clone have shown induction by IL1, TNF and IL6 in a permanently transfected murine fibroblast line (Woo *et al*, 1987; Edbrooke and Woo, 1989). In addition the importance of an NFκB motif in the promoter of the gene has been demonstrated in mediating induction of transcription by IL1 (Edbrooke *et al*, 1989; 1991). Studies currently underway in this laboratory aim to elucidate the mechanisms governing tissue-specific control of SAA gene expression and the possible involvement of SAA mRNA stabilization in induction by cytokines. The *SAA2ß* promoter was also investigated further in work for this thesis with respect to its activation by IL1 and IL6.

1.3 Mediators of the Acute Phase Response.

1.3.1 Introduction.

Koj first proposed that blood-borne mediator(s) maybe responsible for controlling the APR (Koj, 1974). Efforts to identify these factors led to the so called "cytokine explosion" of the 1980s with the isolation and cloning of many of these molecules. Included in this group of cytokines are the interleukins, TNFs, PDGFs, FGFs, IFNs and CSFs. In this overview the emphasis will be on those which have a pivotal role in modulating the APR, largely IL1 and IL6 but also TNF, IFNγ and other HSFs. These will be examined in terms of the current knowledge of their function and modes of action.

1.3.2 Interleukin 1.

The first cytokine found to induce acute phase genes (Sipe *et al*, 1979), termed SAA-stimulating factor (SAASF), was found to be equivalent to leukocytic pyrogen and lymphocyte activating factor (McAdam *et al*, 1982). Endogenous pyrogen (Atkins, 1960), a comitogen for thymocytes and lymphocytes (Grey *et al*, 1972), leukocyte endogenous mediator, lymphocyte activating factor, mononuclear cell factor, catabolin, osteoclast activating factor and haemopoietin-1 are all activities of the molecules now designated IL1 (reviewed by Dinarello, 1984, 1989; Mizel, 1989; di Giovine and Duff, 1990). IL1 comprises acidic (IL1α, pI 5.0) and basic (IL1ß, pI 7.0) forms of 17.5 kD, processed from a 31kD precursor. cDNAs were originally cloned from human (IL1ß, Auron *et al*, 1984)

and mouse (IL1α, Lomedico *et al*, 1984) and the 7 exon genes obtained later (Clark *et al*, 1986; Furutani *et al*, 1986). The human α and ß forms show little (26%) amino acid homology (March *et al*, 1985) but bind the same receptor(s) (Dower *et al*, 1986; Bird and Saklatvala, 1986) and have the same range of activities, although differential responses are found in individual systems (*e.g.* Katsura *et al*, 1988; Garcia-Welsh *et al*, 1990). IL1 is unusual in not having a classical hydrophobic signal sequence characteristic of secreted proteins, in monocytes a specific convertase activity cleaves preIL1ß at the Asp^{116}-Ala^{117} site to yield the 17.5kD molecule (Kostura *et al*, 1989). The classical ER-Golgi route is not used in IL1 secretion and IL1ß is contained in intracellular vesicles in monocytes which protect it from protease digestion (Rubartelli *et al*, 1990). The secretory process appears to be inefficient in that large amounts of IL1 remains cell associated (Auron *et al*, 1987). A significant fraction of IL1 may be secreted as the 31kD form, and proteases released at the site of inflammation (elastase, cathepsin G, collagenase) are capable of processing this to a biologically active form (Hazuda *et al*, 1990). Molecules with IL1 immunoreactivity down to 2kD have been identified some with biological activity, others may act as inhibitors (see Auron *et al*, 1987 for map of potential cleavage sites). Although the main source of IL1 is cells of the monocyte/ macrophage lineage, vascular endothelial cells and smooth muscle cells, synovial fibroblasts, keratinocytes and kidney epithelial cells are among other producers.

Table 1.5 Systemic and Local Effects of IL1.

Systemic	Local
Induction of:	Endothelium:
fever	Induction of:
slow wave sleep	PAF
ACTH secretion	PG
Increased APPs	procoagulant
Reduced plasma Fe and Zn	adhesiveness
Immune response:	Activation of:
Activation of B/T Cells	osteoclasts
Cytokine network:	chondrocytes
Induction of cytokines, e.g. IL1, TNF, IL6, *gro*, IL8	Collagenase induction
Synergy with e.g. TNF, IL6	
Haematopoiesis:	
Synergy with CSFs	

The effects of IL1 can be divided into systemic and local (paracrine/autocrine), these are summarized in table 1.5. Systemic effects include induction of fever, slow wave sleep, reduction of plasma zinc and iron levels and elevation of APPs. Effects on the vascular endothelium include induction of prostaglandins (see Albrightson *et al*, 1985 and references therein) and platelet activating factor, activation of procoagulant activity and

adhesion molecules. IL1 acts in the complex network of inflammatory mediators upregulating cytokines including itself (Dinarello *et al*, 1987) and interacting with the hypothalamus-pituitary-adrenal axis resulting in corticosteroid production (Bernton *et al*, 1987; Berkenbosch *et al*, 1987; Sapolsky *et al*, 1987). The adrenal corticosteroids are immunosuppressive and antiinflammatory blocking the action of several cytokines such as IL2 and IFNγ as well as prostaglandin production. In addition corticosteroids downregulate IL1 production by macrophages and the induction of Ia antigen necessary for antigen presentation (Besedovsky *et al*, 1986). Whereas IL1 downregulates hepatic glucocorticoid receptors (Hill *et al*, 1986, 1988), glucocorticoids have been found to upregulate IL1R on human peripheral blood lymphocytes (Akahoshi *et al*, 1988). Prostaglandins act in a negative feedback pathway reducing IL1 accumulation, probably via a post transcriptional mechanism (Knudsen *et al*, 1986).

There are at least two classes of IL1-R. An 80kD protein on mouse T lymphoma cells could be cross-linked to human IL1 and had a K_D of $0.2\text{-}2x10^{-10}$M. The range of binding sites on different cell lines varied from 27 on human peripheral blood monocytes to $>10^3$ on human and murine fibroblasts (Dower *et al*, 1985). The same receptor could bind both IL1α and IL1ß in various human, murine and porcine cells (Dower *et al*, 1985; 1986; Bird and Saklatvala, 1986; Bird *et al*, 1988). Molecular cloning of the receptor from human and murine T cells demonstrated that the receptor was a member of the Ig superfamily (Sims *et al*, 1988, 1989). The human protein has a 319 amino acid extracellular domain with 3 Ig folds and extensive glycosylation, a 20 amino acid membrane spanning region and a 213 amino acid cytoplasmic domain (Sims *et al*, 1989; see figure 1.1). A two chain model was proposed for the IL1R, analogous to the IL2R (Bird *et al*, 1987; Kroggel *et al*, 1988; reviewed by Dinarello *et al*, 1989) following the initial observation of two receptor affinity classes in EL4 cells (Lowenthal and McDonald, 1986). However expression of the cDNA for the 80kD receptor in Cos cells produced two affinity classes (Sims *et al*, 1989).

A 68kD IL1 receptor has been found on B cell lines (Matsushima *et al*, 1986; Horuk *et al*, 1987; Bensimon *et al*, 1989) with the 80kD receptor being present on T cells and fibroblasts (Chizzonite *et al*, 1989; Bomsztyk *et al*, 1989). Cell-line specific patterns of IL1-R expression were found in human B cell lines with 65, 68, 70 and 80kD binding species (Benjamin *et al*, 1990). Other cross-linking studies have detected proteins ranging from 60 to 200 kD. Hence IL1 effects maybe mediated via different cell-type specific mechanisms including different modes of action for IL1α and ß.

Molecules other than IL1α and ß binding the IL1-R have been characterized recently, including the IL1 receptor antagonist (IL1ra). Hannum *et al* (1990) purified 3 IL1 inhibitors from IgG stimulated human monocytes which differed only by the glycosylation status. Expression of IL1ra mRNA, which is homologous to IL1ß and to a lesser extent to IL1α, is induced by stimuli similar to those that induce IL1 (Eisenberg *et al*, 1990). Other potential inhibitors acting after IL1 synthesis appear to exist (reviewed

by Larrick, 1989) and both recombinant and "naturally" occurring shed IL1 receptors have inhibitory activity (Maliszewski *et al*, 1990; Symons and Duff, 1990). Thus the presence of specific inhibitors of IL1 adds a further level of complexity to the control of IL1 activity in addition to those which occur at the level of transcription and translation (for example, see Fenton *et al*, 1987; Nishida *et al*, 1988; Elias *et al*, 1989b).

Some controversy surrounds a signal transduction pathway for IL1 and a number of mediators have been implicated (see table 1.6). This subject is covered well by the recent debate of Mizel (1990) and O'Neill *et al* (1990a). It seems likely that IL1 can operate by activating a number of transduction pathways in different cell types and maybe a result of differing receptors expressed on those cells. In addition IL1 can activate a number of transcription factors including NFκB (Osborn *et al*, 1989; Espel *et al*, 1990), AP-1 components (Colotta *et al*, 1988; Muegge *et al*, 1990), c-*myc* (Kovaks *et al*, 1986; Lin and Vilcek, 1987) and NF-IL6 (Isshiki *et al*, 1990). Thus induction of specific gene transcription by IL1 maybe orchestrated through distinct sets of *trans*-activating factors in various tissues.

Table 1.6 The IL1 Signal Transduction Debate

Mediator	References
PT-sensitive G-protein	1
cAMP	2
cGMP	3
Diacylglycerol	4
Protein kinase C	5
hsp27	6
Receptor-mediated endocytosis	7

Examples are shown of possible factors which mediate the IL1 signal following receptor binding. PT = pertussis toxin; hsp = heat shock protein. References: 1) Chedid *et al* (1989); O'Neill *et al* (1990b). 2) Shirakawa *et al* (1988; 1989), Zhang *et al* (1988), Chedid and Mizel (1990); Renkonen *et al* (1990) but see also Rodan *et al* (1990); O'Neill *et al* (1990a). 3) Pfeilschifter and Schwarzenbach (1990). 4) Rosoff *et al* (1988); Wijelath *et al* (1988); Kester *et al* (1989). 5) Ostrowski *et al* (1988); Munoz *et al*, 1990) but see Macchia *et al* (1990); Taylor *et al* (1990); Abraham *et al* (1987). 6) Kaur and Saklatvala (1988); Kaur *et al* (1989). 7) Matsushima *et al* (1986); Mizel *et al* (1987); Qwarnstrom *et al* (1988); Grenfell *et al* (1989). IL1 also induces phosphorylation of a number of other cellular proteins (Bird and Saklatvala, 1989; Gallis *et al*, 1989; Hulkower *et al*, 1989)

1.3.3 Tumour Necrosis Factor.

TNF is recognized as a major mediator of shock, inflammation and tissue catabolism. Two forms of TNF (α and ß/cachectin and lymphotoxin) are known which show 30% homology and can compete for the same cell surface binding sites (Aggarwal *et al*, 1985). TNFα was originally identified as a protein which could induce necrosis of

tumours in mice (Carswell *et al*, 1975). TNFα is produced largely by activated macrophages with LPS being the most potent stimulator, whereas TNFß is synthesized by activated lymphocytes (as is TNFα following PMA/A23187 costimulation; Cuturi *et al*, 1987). The genes for both forms of TNF are found in the MHC region of chromosome 6 in man (Spies *et al*, 1986). TNFα is a 17kD non-glycosylated protein encoded by a 4 exon gene. The 157 residue mature protein is cleaved from a 233 residue precursor which like IL1 lacks a typical hydrophobic signal sequence (Pennica *et al*, 1984; Shirai *et al*, 1985; Wang *et al*, 1985).

Despite being structurally distinct TNFα shares many biological effects with IL1 including the induction of a subset of APPs (Perlmutter *et al*, 1986a; Sipe *et al*, 1987; Koj *et al*, 1987; Woo *et al*, 1987) and both exert hormone-like activities on a number of cell types including endothelial cells, fibroblasts and leukocytes (reviewed by Le and Vilcek, 1987). Like IL1, TNFα can also act as an endogenous pyrogen, injection into rabbits causes fever as well as induction of IL1 and hypothalamic PGE_2 (Dinarello *et al*, 1986). TNF activates neutrophils, stimulating the expression of adhesion molecules on both neutrophils themselves and on endothelial cells (Pober *et al*, 1986) as well as secretion of CSF and chemotactic factors by endothelial cells (see Seelentag *et al*, 1987; Strieter *et al*, 1989). In addition to the secreted 17kD TNF molecule a non-secreted 26kD integral membrane form which retains the 76 amino acid leader sequence has been identified (Kriegler *et al*, 1988). This suggests that the the transmembrane protein must be cleaved to release the 17kD protein into the serum. However the larger form is cytotoxic via direct contact of the cells on which it is expressed with target cells (Perez *et al*, 1990). Some activities of TNF are listed in table 1.7 and are described in the reviews of Le and Vilcek (1987); Beutler and Cerami (1988a; 1988b); Old (1988); and Tracey *et al* (1989).

Table 1.7 **Some Activities of TNF.**

Pyrogenic
Cachectic
Tumouricidal
APP induction
Activation of neutrophils
Increased vascular adhesiveness
Induction of procoagulant activity
Osteoclast activation
Suppression of adipose-specific genes
Differentiation of leukaemic cells
Induction of IL2-R

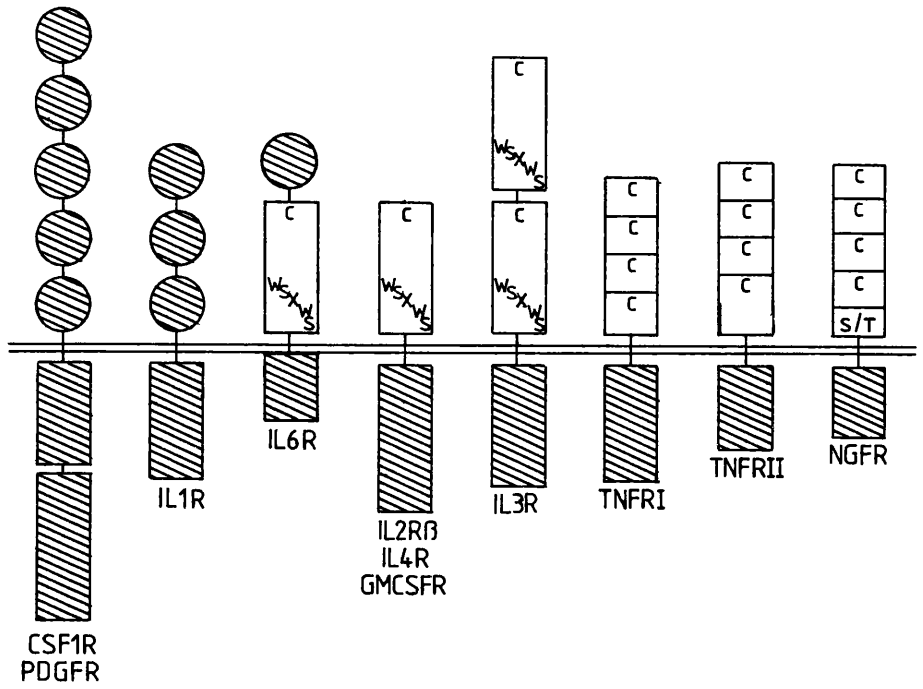


Figure 1.1 Structural Relationship of Cytokine Receptors.
A schematic representation of cytokine receptor groups showing immunoglobulin-like domains (shaded circles) and other features. Cysteine-rich subdomains (C) occur in a number of the receptors, these are within 30-40 amino acid repeating units in TNF and NGF receptors. The WSXWS box is found in the juxtamembrane region of multiple cytokine receptors (see Bazan, 1990). S/T = serine/threonine-rich domain. The scale is arbitrary and the shaded cytoplasmic domains non-homologous.

A receptor was identified for TNF which bound both TNFα and ß and was endocytosed following binding (Baglioni *et al*, 1985; Aggarwal *et al*, 1986; Nawroth *et al*, 1986). In addition both high (K_D=2.6x10^{-13}M) and low (K_D=1.5x10^{-10}M) affinity binding sites were found on the same cell type (Imamura *et al*, 1987). Recently a number of reports on molecular cloning of TNF-Rs have shown that there are at least two classes. The type I (55kD) TNF-R has a 223 amino acid C-terminal cytoplasmic domain, a single transmembrane span of 21 amino acids and a 182 amino acid extracellular domain containing 4 cysteine-rich repeat sequences which show homology to the nerve growth factor (NGF) receptor (Gray *et al*, 1990; Loetscher *et al*, 1990; Nophar *et al*, 1990; Schall *et al*, 1990). The type II TNF-R (68kD) also has a cysteine-rich extracellular domain homologous to CD40 (and NGF-R) (Hellu *et al*, 1990; Smith *et al*, 1990). The homology between the type I and II receptors is no greater than between other members of the family (ie. NGF-R, CD40 etc.). The cysteine-rich sequences identified are characteristic of members of the cytokine receptor superfamily (see figure 1.1). Two distinct forms of TNF binding proteins, TBPI and TBPII of 30kD have been isolated from the urine and

serum of cancer patients (Engelmann *et al*, 1990) and appear to be shed forms of the type I and II receptors. TNF activity can therefore be modulated by prevention of functionally effective receptor binding although by a different mechanism than IL1. Latent receptors for TNF exist in the plasma membrane which are upregulated on TNF binding (Han *et al*, 1990), these may represent distinct receptor types or potentially second chains.

Studies on TNF signal transduction mechanisms have not produced a clear consensus, again this maybe accounted for by separate pathways being used by different receptors in various tissues. TNF-R is associated with a 200kD cell surface antigen called Fas which may mediate the cytolytic activities of TNF (Yonehara *et al*, 1989). There is some disagreement over the potential involvement of PKC and PKA pathways in transducing the TNF signal (see Zhang *et al*, 1988; Brenner *et al*, 1989; Schutze *et al*, 1990). IL1 and TNF appear to activate the same kinase (or inactivate a phosphatase/kinase inhibitor) as identical phosphorylation specificities occur in fibroblasts, namely increased serine phosphorylation of hsp27 and EGF-R (Kaur *et al*, 1989; Bird and Saklatvala, 1989).

A number of transcription factors are induced by TNF varying according to cell type. For instance *fos* and *jun* are induced in human fibroblasts (Brenner *et al*, 1989) as is *myc* (Lin and Vilcek, 1987). In endothelial cells TNF increases AP-1/c-*jun* but not *fos* or *myc* (Dixit *et al*, 1989). TNFα can also induce NFκB activity (Osborn *et al*, 1989; Lowenthal *et al* 1989; Duh *et al*, 1989; Israel *et al*, 1989; Pessara and Koch, 1990) occurring at low receptor occupancy in the promyelocytic cell line HL60 (Hohmann *et al*, 1990). A novel TNF responsive transcription factor, NFGMa, which binds to regulatory elements in haemopoietic growth factor genes such as GM-CSF, G-CSF, IL3 and IL5 has recently been identified (Shannon *et al*, 1990).

1.3.4 Interleukin 6.

IL6 has been identified as a major mediator of the APR and shares biological properties with both IL1 and TNF. Among the cytokines IL6 is probably the molecule for which the most properties were defined before the realization that these were all the pleiotropic actions of a single substance. Historically IL6 was probably first recognized as the T cell replacing factor from T cell supernatants which induced Ig secretion by B cells (Schimpl and Wecker, 1972). The same activity has been described as IFNß$_2$ (Weissenbach *et al*, 1980), 26kD factor (Haegemann *et al*, 1986), B cell differentiation factor/B cell stimulatory factor 2 (Hirano *et al*, 1984), mouse plasmacytoma growth factor (Nordan *et al*, 1987), human hybridoma/plasmacytoma growth factor (vanDamme *et al*, 1987), T cell differentiation factor (Takai *et al*, 1988) and hepatocyte stimulating factor (Gauldie *et al*, 1987). May *et al* (1988) proposed that this molecule with diverse activities be called "hippocratin... in honour of the Greek physicians of antiquity who first described the AP alterations in the properties of the blood", however a congress in 1988 agreed on the designation IL6 (Sehgal *et al*, 1989).

IL6 is a 184 amino acid molecule derived from a 212 amino acid precusor and secreted by a large nuber of cell types including monocytes, fibroblasts, endothelial cells, T and B lymphocytes, glial cells, keratinocytes and human articular chondrocytes (see Van Snick, 1990). Five different molecular weight IL6 molecules from 23-30kD are seen on denaturing PAGE due to differential glycosylation (May *et al*, 1988). The functions described for IL6 implicit in the activites attributed to it above are diverse. These include induction of differentiation of B cells to high level Ig-producing cells (IgM, IgG and IgA, see Kishimoto and Hirano, 1988) and growth factor activity for T cells. The induction of secreted and membrane bound forms of IgG_1 by IL6 involves effects on transcriptional and mRNA stabilizing processes (Raynal *et al*, 1989). Although not a growth factor for normal B cells, IL6 can act as one for transformed B cells and acts synergistically with other cytokines such as IL3 in enhancement of proliferation of haematopoietic progenitor cells by reducing the G_0 period (Ikebuchi *et al*, 1987). The creation of transgenic mice carrying the human IL6 gene caused massive plasmacytosis, increased megakaryocyte production in bone marrow and development of mesangio-proliferative glomerulonephritis (Suematsu *et al*, 1989). Interactions of IL6 with other cytokines include the downregulation of LPS induced TNF production by monocytes (Aderka *et al*, 1989) and the synergistic activation of T cells with IL1 (Houssiau *et al*, 1988). Like IL1 and TNF, IL6 can act as a pyrogen producing monophasic fever in rabbits (Helle *et al*, 1988).

Table 1.8 **Selected Effects of IL6.**

Induces:

- Differentiation of B cells
- Activation of T cells (with IL1)
- APPs
- Neural cell differentiation (NGF-like activity)
- Mesangial cell growth
- ACTH release

B cell hybridoma/plasmacytoma growth factor

Competence factor for haematopoiesis (with IL3 etc.)

Some of the activities ascribed to IL6 are shown with information derived from references described in the text. Induction of ACTH release by IL6 in rats was reported by Naitoh *et al* (1988) and NGF-like activity by Satoh *et al* (1988).

IL6 appears to be unique among the cytokines investigated in inducing all APPs studied to some extent whereas IL1 and TNF only activate a subset and are inhibitory in some cases (see table 1.2). It has been suggested that IL1 and TNF induce APPs via

autocrine induction of IL6 however in rats *in vivo* IL6 acts as an exocrine hormone (Gauldie *et al*, 1990). Clearly induction of IL6 in other cell types by IL1 and TNF leads to enhancement of the APR. For further discussion of IL6 actions see the following reviews: Wong and Clark, 1988; Kishimoto, 1989; Akira *et al*, 1990; Fey and Gauldie, 1990; Sehgal, 1990; Van Snick, 1990).

A human IL6-R has been cloned from the natural killer cell line YT, expression of this cDNA in the IL6-R negative Jurkat T leukaemic cell line gave binding characteristics consistent with high (10^{-11}M) and low (10^{-9}M) affinity binding sites (Yamasaki *et al*, 1988). The predicted protein is 468 amino acids long with a 19 amino acid signal peptide and a 90 amino acid Ig-like domain, thus placing it in the Ig-superfamily (including CSF1-R, PDGF-R, IL1-R) but has no intracellular tyrosine kinase domain like other growth factor receptors. The IL6-R is upregulated by dexamethasone on hepatoma cells (Rose-John *et al*, 1990) and epithelial cells (Snyers *et al*, 1990) which could explain the requirement for glucocorticoid in AP induction by cytokines in several systems. Although several different molecular weight species of IL6-R are detected by affinity cross-linking besides the expected 80kD these are thought to result from various multimers and association with other proteins (Rose-John *et al*, 1990; 1991).

IL6 receptor binding triggers association with the membrane protein gp130 which maybe a signal amplifier and transducer. Association between the two molecules is extracellular as soluble IL6-R/IL6 complex lacking the transmembrane and cytoplasmic portions could associate with gp130 (Taga *et al*, 1989). Transfection of IL6-R negative cells with IL6-R cDNA produces mainly low affinity binding sites, co-transfection with gp130 cDNA produces more high affinity sites although gp130 does not directly bind IL6 (Hibi *et al*, 1990). Following treatment of IL6-dependent B cell hybridomas with IL6 a 160kDa cytoplasmic protein is rapidly tyrosine-phosphorylated followed by a second protein kinase activity distinct from known kinases (Nakajima and Wall, 1991). In addition CD40 has also been shown to be phosphorylated following IL6 treatment of these cells (Clark and Shu, 1990). Further studies will show whether these events occur in response to IL6 in other cell types.

1.3.5 Other Acute Phase Mediators.

In addition to the cytokines described above other modulators of the APR have also been investigated. A number of HSFs (I-III) are secreted from the COLO16 squamous carcinoma cell line and have been found to regulate distinct sets of APPs. In H-35 cells, HSFI increased C3 and Hp expression whereas HSFII increased ACH, CPI, AT, FBN and Hx. Each factor was 10-100 times less effective at stimulating factors of the other set (Baumann *et al*, 1987a). HSFII and IL6 stimulate the same set of APPs but have distinct structural properties (Baumann *et al*, 1989). HSFIII is a 39kD glycoprotein, structurally and functionally indistinguishable from leukaemia inhibitory factor (LIF) (Baumann and Wong, 1989) which induces macrophage differentiation of murine M1

myeloid leukaemic cells and inhibits differentiation of embryonic stem cells (Gearing *et al*, 1987).

Perlmutter *et al* (1986c) and Miura *et al* (1987) found that IFNγ could regulate complement genes. Although IFNγ can directly activate some APPs one of its major roles appears to be in modulating the activity of other cytokines. Several studies have demonstrated the upregulation of TNF-R by IFNγ (Aggarwal *et al*, 1985; Ruggiero *et al*, 1986; Tsujimoto *et al*, 1986), synergizing with TNF and IL1 for this activity (Chen *et al*, 1987) and with TNF in induction of CSFs (Lu *et al*, 1988). IFNγ also enhances the LPS stimulation of IL6 activity in human monocytes (Cheung *et al*, 1990) and directly stimulates IL6 mRNA synthesis in human FS-4 fibroblasts (Kohase *et al*, 1987).

Transforming growth factor ß (TGFß) a 25kD dimer has recently been shown to have effects on AP gene expression (Morrone *et al*, 1989; Bereta *et al*, 1990; De Li Shi *et al*, 1990). Many cell types synthesize TGFß and its receptor has a wide tissue distribution. TGFß tends to antagonize the effects of other cytokines having strong antimitogenic properties on many cells in culture including hepatocytes and fibroblasts. The relevance of this molecule in inflammation has been demonstrated by its release from platelets (Assoian and Sporn, 1986) and activated T cells (Derynk *et al*, 1985). As such TGFß provides a feedback control on clonal expansion as it blocks the mitogenic activity of IL2 on T cells. TGFß also suppresses ACTH induced steroidogenesis (see Sporn *et al*, 1986 for review). An element has been identified in a number of TGFß inhibited genes (TGFß inhibitory element, TIE = GAGTTGGTGA) which is required for inhibitory effects and binds a protein complex containing *fos* (Kerr *et al*, 1990)

An increasing number of mediators which are important during the APR have been characterized, future work will further elucidate the complex interactions which occur within the cytokine network which governs the fine tuning of the inflammatory response and APR.

1.4 Eukaryotic Gene Structure.

1.4.1 Evolution of Gene Families.

A feature distinguishing eukaryotic genes from those of lower organisms is the presence of intervening sequences which break up the coding regions. Introns maybe by-products of the processes of recombination which bring genes together (Gilbert, 1978) and appear to be useful in the formation of genes allowing combinatorial assemblies of smaller minigenes to form a mosaic encoding a larger protein (Gilbert, 1985). This modular assembly model has gained support from the structure of a number of genes including the low density lipoprotein receptor (LDL-R) which contains exons clearly related to a complement C9 exon and exons from the epidermal growth factor precursor (Sudhof *et al*, 1985a; 1985b). Structures such as the Ig fold are often found encoded by single exons and are repeated to varying extents in different genes.

Protein coding regions may have originated by the duplication of an underlying motif on multiple occasions giving rise to blocks of repetitive sequence which then diverged from one another to give the present day structure (Ohno, 1984). The 5 subdomains from which the albumin and α-foetoprotein (αFP) genes were generated by triplication contain nucleotide sequence homologies which suggest the entire αFP gene was assembled from a 27bp repeat (Alexander *et al*, 1984). 42nt repeats are also identifiable as the basis for the mouse proline-rich protein gene family (Ann *et al*, 1988). Simple sequences such as these could expand or contract by unequal cross-over or slippage during replication (Tautz and Renz, 1984), a process which results in the continual loss or gain of direct repeats. Generation of polymorphism by slippage in repeated regions appears to be responsible for the differences in the *period* genes between species of *Drosophila* (Dover 1989).

Members of a gene family have homologous exon/intron structure and nucleotide sequence and may belong to a superfamily comprising genes with homology in a domain or domains (the immunoglobulin fold, for example) from different families (Doolittle, 1980). It is assumed that all genes were originally present as a single copy and identical in a population (Ohta, 1988), and with the exception of the production of the first tandem pair of genes, gene families can expand or contract by unequal but homologous crossing-over. This produces an increased copy number on one chromosome and a reduced copy number on the other. Duplication to produce the first pair of genes could occur by unequal cross-over between repetitive sequences. For example the human α_1AGP genes are thought to have duplicated by unequal homologous cross-over between Alu repeats flanking the genes (Merritt *et al* 1990). Alternatively reverse transcription of an RNA product from a gene produces DNA copies which maybe inserted back into the genome (retroposition). Such a mechanism was probably responsible for the duplication of the human phosphoglycerokinase genes (McCarrey, 1990). Gene families such as metallothionein (Peterson *et al*, 1989), amylase (Wiebauer *et al*, 1985), adenylate deaminase (Monisaki *et al*, 1990) and crystallin (Lubsen *et al*, 1988) have all demonstrably arisen from a primordial gene. Whereas many duplications involve the gene and a small surrounding region, some such as the murine *Antennapoedia* class homeobox genes were derived by duplication of complete gene clusters (Schughart *et al*, 1989). Although several gene families appear to be in a steady state with respect to copy number and diversity the differential organization of genes which has arisen since mammalian radiation, for example globin (Zimmer *et al*, 1980), interferons (Allen and Fantes, 1980), T cell receptors (Arden *et al*, 1985), haptoglobin (McEvoy and Maeda, 1988) and crystallin (Lubsen *et al*, 1988) genes shows that the process of gene duplication is an on going process tested by natural selection.

Genes within a family often display concerted evolution. For instance regions of homology between adjacent ß-like globin genes on one chromosome are not reflected on the homologous chromosome i.e. γ^A and γ^G genes on the same chromosome are more

homologous than allelic γ^As (Slightom *et al*, 1980). Similar findings were apparent in mouse Ig genes where homologous segments of non-allelic genes were not shared by allelic genes (Schreier *et al*, 1981). Smith (1976) first hypothesized that homogenization of a gene family could occur by unequal sister chromatid exchange, a model most applicable to reiterated sequences with no intervening DNA. Thus gene conversion, defined as the non-reciprocal transfer of genetic information from one gene to another, has been proposed as a model to account for the occurrence of concerted evolution in gene families (although generation of diversity is also possible within this model) (Baltimore, 1981).

Two molecular mechanisms have been proposed to explain gene conversion: single-strand break with heteroduplex correction, and repair of double-strand breaks (reviewed by Kourilsky, 1986). In the former model a single stranded break occurs in the donor gene, the 3' end invades an homologous duplex with the formation of heteroduplex DNA. This is followed by repair (see figure 1.2). If mismatches occur between the two strands of the heteroduplex then mismatch repair will occur, this can occur randomly in blocks, thus depending on the template used patchworks may occur. In essence the sequence is conserved as all the information within it derived from the original genes, however the formation of patchworks generates diversity. The double strand repair break model does not allow for the formation of patchworks. A double strand break occurring at recombination is enlarged by exonucleases to leave 3' single strands, one 3' end invades the homologous duplex creating a D loop which is enlarged by repair synthesis until it can anneal with the other 3' end. Repair synthesis from the second 3' end followed by gap repair and resolution of the two Holliday junctions formed results in conversion of the strand in which the original break occurred (Szostak *et al*, 1983). Gene conversion between 2 genes could occur between alleles on homologous chromosomes, tandem genes interacting on sister chromatids or a pair of related genes anywhere in the genome and could occur at mitosis or meiosis.

Studies of gene conversion events in a number of gene families (e.g. primate foetal γ-globin genes, Fitch *et al*, 1990) have led to the recognition of several features:
i) Conversion events are restricted to regions with high sequence homology.
ii) Although theoretically conversion can occur in either direction, polarity is often evident with one gene being dominant.
iii) Gradients occur apparently dependent on the location at which initiation of conversion occurs (Eickbush and Burke, 1986; McCormack and Thompson, 1990).
iv) Some sequences involved in conversion accumulate changes more rapidly than expected: this may be due to the patchwork formation described above.
v) Certain repetitive elements appear to be involved in initiating/ terminating conversion.

In order to escape gene conversion a sequence must evolve quickly to reduce sequence identity and/or a lose the ability to initiate concerted evolution (by changes in sequences promoting sequence exchanges) (Walsh, 1987). Processes such as patchwork

formation, replication slippage in repeated regions, recombination between repetitive elements and insertion of transposable elements (Finnegan, 1989) can all generate diversity. If a conversion event has a selective disadvantage then non-converted sequences will be selected for and the genes will diverge.

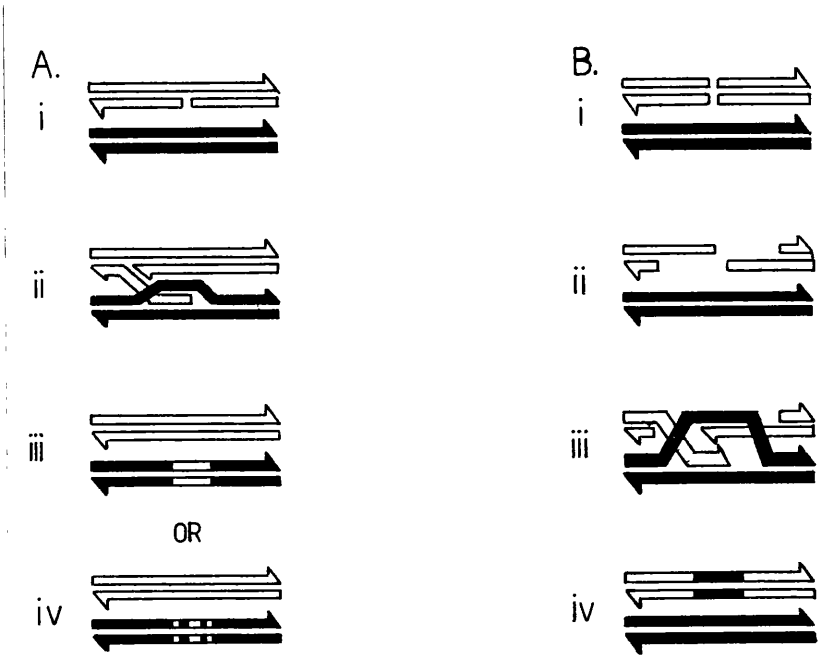


Figure 1.2 Molecular Mechanisms for Gene Conversion.
A) The single strand break model. A break occurs in one strand with the invasion of an homologous duplex by the free strand and the formation of an heteroduplex. Repair synthesis then occurs which produces the result in (iii) if the invading strand is used as a template, alternatively patchworks may occur (iv) depending on the template used.
B) Nuclease activity produces 3' overhangs (ii) from a double strand break which is repaired using the homologous duplex as template as shown. In this case the molecule in which the break occurs is converted.

1.5 Transcriptional Control of Gene Expression.

1.5.1 Transcriptional Regulation.

Studies on gene regulation have led to the paradigm that a gene remains inactive until transcription factors become available which bind DNA at specific *cis*-acting sequences and interact (either directly or through other mediators) in a cooperative fashion with the polymerase complex to initiate transcription. The combinatorial effects of transcription factors at a promoter are thought to result in the correct spatial and temporal expression of a gene. In the following sections the nature of these intreactions and current knowledge of transcriptional activation will be reviewed, focussing on the properties of transcription factors and their modes of activation. Studies on NFκB important for SAA gene regulation will be discussed in greater detail.

1.5.2 Enhancers and Promoters.

In recent years 3 classes of *cis*-acting sequences have been identified (Maniatis *et al*, 1987). Promoters function in the immediate vicinity of a gene and are required for accurate and efficient initiation whereas enhancers modulate transcription in a position and orientation independent manner and maybe several kb 5' or 3', (enhancers in the Ig genes are located in the introns between the joining (J) and constant (C) gene segments or 3' of the C segments; see Calame, 1989). In practice the distinction between enhancers and promoters may be less clear, for example glucocorticoid response elements (GREs) can function as both (Schatt *et al*, 1990). The third category of *cis*-acting sequence, repressors act in a position and orientation independent manner but suppress transcription (Maniatis *et al*, 1987). Specific combinations of *trans*-acting factors binding to these sequences are required for the correct spatial and temporal control of gene expression.

1.5.3 Transcriptional Activators.

Characterization of transcriptional activators (see Ptashne, 1988) has shown that they are: i) modular, with separable domains carrying out particular functions such as DNA binding and transcriptional activation (see Frankel and Kim, 1991);

ii) promiscuous, with for example yeast transcription factors being functional in mammalian cells provided the specific *cis*-acting sequence is present;

iii) able to function cooperatively in induction: when binding sites for 2 factors are present the activation of a nearby promoter is often greater than the sums of the individual activations;

iv) maintained in an inactive form until required.

The modular and promiscuous nature of transcription factors was clearly shown in the first "domain swap" experiment where the DNA binding domain of a yeast activator GAL4 was swapped with that of a bacterial repressor, in yeast this activated transcription of a gene with the LexA operator upstream (Brent and Ptashne, 1985). When a truncated GAL4 protein containing the N-terminal DNA binding domain but not the activating region was used DNA binding occurred but without transcriptional activation (Keegan *et al*, 1986).

1.5.4 Transcription Factor Domains: DNA Binding, Dimerization, and *Trans*-activation.

A number of domains have been found to be conserved among groups of transcription factors (see Mitchell and Tjian, 1989; Latchman, 1990). These include the homeodomain, a 61 amino acid region containing a helix-turn-helix homologous to that found in certain bacterial repressor proteins. This domain was first found in the genes controlling morphogenesis in *Drosophila* (Scott and Weiner, 1984) and has subsequently been found throughout phylogeny (Gehring, 1987). The homeodomain alone is sufficient for sequence specific DNA binding of these proteins as monomers, but sequences outside the homeodomain affect DNA binding specificity (Hayashi and Scott, 1990). Mammalian proteins such as Pit-1, Oct-1 and Oct-2 and the *Caenorhabditis elegans* factor unc-86 contain a particular class of homeodomain together with a 75 amino acid region upstream

which together are defined as the POU domain (see Robertson, 1988). Each of the POU proteins uses the POU specific domain to bind to DNA, however the relative contributions of this and the homeodomain to DNA binding is at present unknown.

A second class of DNA binding motif is the zinc finger originally found in *Xenopus* TFIIIA (reviewed by Klug and Rhodes, 1987). In this model a pair of cysteines and a pair of histidines are tetrahedally coordinated about a zinc ion with the formation of a finger by the 12 amino acids between the cysteine and histidine pairs. Proteins of the C_2H_2 type usually have multiple fingers which interact with the major groove of the DNA and are found in factors such as Sp1 (Kadonaga *et al*, 1987). A second type of finger consisting of 4 cysteines coordinating a zinc ion is found in a number of transcription factors such as the steroid/ thyroid hormone receptors. These C_4 type proteins have only 2 fingers which bind a palindromic sequence (such as the GRE). Although the fingers bind DNA the determinants of DNA binding specificity reside in the base of the finger (Mader *et al*, 1989). The binding sequences for the glucocorticoid receptor (GR) and oestrogen receptor (ER) are related, Danielsen *et al* (1989) showed that altered activation occurred in "finger swap" experiments between the 2 proteins with the ability to discriminate between the GRE and ERE residing in 2 amino acids located in the "knuckle" of the first zinc finger .

Landschulz *et al* (1988) found a 30 amino acid homology between the liver-enriched factor C/EBP and the proto-oncogene product Myc predicted to form an α-helix with a leucine at every seventh residue. They proposed that leucine side chains extending from one α-helix interdigitate with those from a similar region on another molecule forming a leucine zipper. The Jun family of proteins also contains this motif, mutagenesis of c-Fos demonstrated the necessity of the leucine repeat for dimerization with c-Jun. Dimerization results in the appropriate juxtaposition of adjacent basic amino acid regions for DNA binding (Turner and Tjian, 1989; Gentz *et al*, 1989). Both basic binding domains in a heterodimer must be intact otherwise no DNA binding occurs (Smeal *et al*, 1989). As well as C/EBP and Jun families ATF/CREB proteins also contain the bZIP domain (Hoeffler *et al*, 1989).

A further dimerization domain is the helix-loop-helix which also has an associated basic DNA binding domain (Davis *et al*, 1989). These bHLH proteins include E12 and E47 (Murre *et al*, 1989a), myoD, myf5 and myogenin. The HLH comprises 2 short amphipathic helices with hydrophobic residues at every third or fourth position, the intervening loop usually contains helix breaking residues. Proteins such as Myc and Max with which it dimerizes contain both a HLH and ZIP domain, the integrity of which is required for interaction (Blackwood and Eisenmann, 1991).

Dimerization of transcription factor molecules increases the repertoire of proteins which can bind DNA with differing affinities and binding specificities. This increased complexity may contribute to the fine tuning of the transcriptional response. For example MyoD/E12 or MyoD/E47 bind better to the κE_2 site than homodimers of these species

(Murre *et al*, 1989b). Heterodimers of Fos or CREB with Jun show differing affinities for the related AP-1 and CRE sites (Benbrook and Jones, 1990; see Jones, 1990).

Many transcription factors whose structures have been delineated have no known structural motifs. These include NFκB (although the *rel* homologous domain maybe involved in dimerization), AP-2, serum response factor (SRF) and CTF/NF-1. Recently identified homologies between transcription factors include the *fork head* DNA binding domain of the rat liver-enriched nuclear factor HNF-3A and the *Drosophila* homeotic gene *fork head* (Weigel and Jackle, 1990). The ETS domain consists of an 85 amino acid region found in a number of DNA binding proteins including the murine homologue of the avian *ets*-1 proto-oncogene and related proteins *PU.1* and *Drosophila E74* (Karim *et al*, 1990). In addition sequences related to those found in the high mobility group genes (the HMG box) are found in the polI factor UBF (Jantzen *et al*, 1990), *SRY* the candidate sex-determining gene (Gubbay *et al*, 1990; Sinclair *et al*, 1990) and the T cell specific factor TCF-1 (van de Wetering *et al*, 1991).

Transcriptional activating domains were originally identified as acidic regions with subunits that acted in an additive manner. When these were fused directly to a DNA binding domain a functional activator was produced (Hope *et al*, 1988). It was even possible to convert the yeast inhibitor of GAL4, GAL80 into an activator by the insertion of an acidic region (Ma and Ptashne, 1988). These acidic regions have been reported to interact directly with TFIID, for example, when VP16 an activator with an acidic region is attached to an affinity column factor(s) are bound in mammalian cell extracts with the properties of TFIID (Stringer *et al*, 1990).

Although the activation domains of activators such as GAL4, AP-1 (Bohmann *et al*, 1987) and one of the 2 activation domains of GR (Hollenberg and Evans, 1988) are acidic, those of several other activators do not fit this category. For example Sp1 has 4 activation domains of which the 2 most potent are glutamine (Q)-rich with similar regions being found in the *Antennapoedia*, *Ultrabithorax* and *Zeste* proteins of *Drosophila*, Oct-1, Oct-2, Jun, AP-2 and SRF (Mitchell and Tjian, 1989). CTF/NF-1 has a proline-rich region also found in AP-2, Jun, Oct-2 and SRF. Thus activators may not have common mechanisms for interacting with the transcriptional machinery and the existence of transcriptional adaptors has been implied.

1.5.5 Transcriptional Adaptors.

Insight has been gained into the mechanism of transcriptional activation through "squelching" experiments. In these studies a high concentration of one activator, Y, inhibits transcription induced by factor X, from a promoter lacking a binding site for Y, presumably by sequestering a common target protein also required by X. In the first squelching experiments when GAL4 was expressed at high levels in yeast cells transcription was inhibited from genes lacking GAL4 binding sites (Gill and Ptashne, 1988). The common target protein could be TFIID or polymerase, however in the squelching experiments of Kelleher *et al* (1990) using the DNA binding domain of GAL4

fused to the acidic activation domain of VP16, squelching of a promoter containing a T-rich sequence (for T-rich binding factor) occurred which could not be relieved by addition of TFIID or polymerase but was relieved by addition of other (unidentified) yeast factors, indicating the requirement for another component. Evidence had previously been provided for such transcriptional adaptors with the observation that cloned TFIID could not increase transcription when Sp1 was added even though basal transcription occurred, however use of crude TFIID preparations allowed induction (Pugh and Tjian, 1990). Berger *et al* (1990) also found evidence for transcriptional adaptors via GAL4-VP16 squelching from the T-rich promoter. Although proposed that these adaptors somehow mediate interaction between acidic activating regions and TFIID, they may work by some other mechanism such as connecting TFIID to polymerase, this would be consistent with the finding that VP16 binds directly to TFIID (Stringer *et al*, 1990). Direct evidence for adaptors has been obtained with the purification of a fraction from yeast cells which was necessary for stimulation of transcription *in vitro* of GAL4-VP16 and GCN4 (Flanagan *et al*, 1991). For further discussion on adaptors see Ptashne and Gann (1990) and Lewin (1990).

The foregoing relates to transcriptional activators which interact to induce transcription through "acid blob" regions. Experiments have been carried out to determine common cellular targets of activators lacking acidic activation regions (such as E1a and human oestrogen receptor transactivation functions) and those with acidic regions. The results suggest there may be strings of intermediary proteins required for interaction with the transcriptional machinery with different transactivators interacting at different points along the chain (see Martin *et al*, 1990; Tasset *et al*, 1990). The mechanism of action of other activators with non-acidic regions such as Sp1 and CTF/NF-1 is not clear at present, these may have adaptor molecules too or may interact with the transcriptional machinery in a different manner.

1.5.6 Activation of Transcription Factors.

Transcription factors can be regulated at transcriptional, posttranscriptional or translational levels and their activity can be modulated by cellular levels or the ability to interact with target molecules (reviewed by Falvey and Schibler, 1991). Many factors are regulated by modification of pre-existing proteins, thus DNA binding of serum response factor (SRF) requires phosphorylation by a casein kinase II-like kinase (Manak *et al*, 1990). Phosphorylation can also inactivate transcriptional activators as is the case for the PKA phosphorylation of the yeast activator ADR1 (Cherry *et al*, 1989). GR is activated by association with its ligand to bind DNA, in the absence of glucocorticoid the receptor is maintained in the cytoplasm where it is associated with hsp90. MyoD1 is also associated with an inhibitor, Id whose levels fall during muscle differentiation (Davis *et al*, 1987). Id has an HLH domain but not an adjacent basic domain and thus sequesters MyoD and prevents DNA binding. Genes activated by heat shock contain a heat shock element (HSE) which binds heat shock factor (HSF). In yeast affinity of HSF for HSE is

unaffected by temperature, however transcriptional activation is dependent on phosphorylation. This is also true of HeLa HSF except that DNA binding activity is also regulated although not by phosphorylation (see Berk, 1989). As described elsewhere dimerization of a factor can radically affect its specificity and activity on a particular promoter. Thus the relative levels of individual molecules which make up the dimers are important.

Other levels of control are also important, for example NFAT-1 which is involved in IL2 gene transcription is synthesized *de novo* following a signal from the T cell receptor, 10-25 minutes prior to IL2 transcription (Shaw *et al*, 1988). GCN4 in yeast is upregulated by increased mRNA translation following amino acid starvation (Fink, 1986). The hepatic nuclear factor HNF1 is regualted at the transcriptional level (Frain *et al*, 1989) whereas the mRNAs for DBP and LAP are found in many cell types but the respective proteins only accumulate in the liver (Mueller *et al*, 1990).

Several factors are controlled at more than one level, for example, AP-1 is activated post-translationally by TPA, an increase in *jun* transcription is also detected, mediated via the AP-1 site. Thus a positive autoregulatory loop prolongs the transient signals generated by PKC (Angel *et al*, 1988). Factors such as NFκB and NF-IL6 which are activated posttranscriptionally also have their mRNAs upregulated following activation thus replenishing diminished stocks (Bours *et al*, 1990; Akira *et al*, 1990).

1.5.7 Transcriptional Repression.

The mechanisms by which specific *cis*-acting sequences and *trans*-acting factors can repress transcription are severalfold and include inhibition of DNA binding (*e.g.* GR, Akerblom *et al*, 1988; Ray *et al*, 1990), blocking of activation functions (*e.g.* GAL80 binds GAL4, Ma and Ptsahne, 1988), and silencing by repressor elements (*e.g.* ε-globin silencer, Cao *et al*, 1989; ß-actin silencer, DePonti-Zilli *et al*, 1988). A detailed analysis of these can be found in recent reviews (Levine and Manley, 1989; Renkawitz, 1990).

Some *trans*-acting factors can act in both a positive and negative manner dependent on the context in which they are active. Among these are Fos which as well as being an activator can inhibit several immediate early (IE) genes (such as c-*fos*, *Egr*-1 and *Egr*-2) by a distinct binding site (the CArG box) and utilizing distinct domains (Gius *et al*, 1990). Dexamethasone can confer both positive and negative regulation via a 25bp element of the mouse proliferin gene which binds the glucocorticoid receptor (GR). The effect requires DNA binding by the GR at the "composite GRE" and also protein-protein interaction with members of the AP-1 family which bind in the same region. The relative composition of the AP-1 (i.e. Jun homodimer, Jun/Fos heterodimer etc.) determines whether control is positive or negative (Diamond *et al*, 1990). More recent evidence shows that AP-1 and GR can functionally antagonize each other by protein-protein interaction a process dependent on hormone binding by the receptor but not necessarily

binding to DNA (Jonat *et al*, 1990; Yang-Yen *et al*, 1990; Schule *et al*, 1990). These findings reveal a new level of complexity in gene control where transcription factors can act in a negative fashion without interacting with DNA.

1.5.8 Other Determinants of Gene Control.

In addition to the levels of gene control mentioned, other determinants of transcription exist, dependent on local configuration such as methylation status and chromatin organization. The importance of structural proteins in controlling transcription was demonstrated by the blockage of *in vitro* basal transcription in the presence of nucleosomes. Addition of GAL4-VP16 along with the nucleosome assembly system allows transcription *in vitro*, thus there seems to be some competition between nucleosomes and for example TFIID which is alleviated by the addition of the transcriptional activator (see Ptashne and Gann, 1990). A yeast mating type locus is locked into a chromatin loop, involving proteins in an associated silencer. The loop configuration may be responsible for switching the gene off (Hofmann *et al*, 1989). It has been observed that tissue specific genes are fully methylated in sperm and the female germ line and remain so through out adult development in tissues where they are not expressed. Housekeeping genes often have associated CpG islands which are unmethylated. Thus it has been proposed that methylation distinguishes inactive and active genes. In some cases there is evidence that methylation can affect interaction of factors with *cis*-acting sequences and repress transcription (reviewed by Cedar, 1988).

1.5.9 NFκB.

NFκB was originally identified as a protein important for the function of the immunoglobulin κ light chain enhancer (Sen and Baltimore, 1986) and was thought to be tissue specific in that it was only constitutively present in mature B cells (Lenardo *et al*, 1987). Subsequent analysis identified an inducible form in diverse cell types present in a covert cytoplasmic form (Nelsen *et al*, 1988). Many genes have been identified which contain sequences homologous to the NFκB site and for which functional significance has been demonstrated. These include acute phase genes such as SAA (Edbrooke *et al*, 1989), factor B (Nonaka and Huang, 1990) and angiotensinogen (Ron *et al*, 1990); cytokine and cytokine receptor genes such as TNFα, IL6, IL2, IL2Rα chain, ßIFN; MHC class I and class II genes, $ß_2$microglobulin as well as viral genes such as HIV, SV40, adenovirus and cytomegalovirus (see below also Lenardo and Baltimore, 1989; and Zabel *et al*, 1991).

Purified NFκB is a 50kD molecule (p50) and was thought to bind DNA as a homodimer in association with two 65kD molecules (p65). Binding of p65 to p50 had no inhibitory effect on DNA binding but was essential for association with IκB the cytoplasmic inhibitor of NFκB (Baeuerle and Baltimore, 1988a; 1988b; 1989). The recent availability of recombinant p65 and p50 has confirmed that p65 binds IκB however it also binds the NFκB motif with these molecules binding as homodimers or heterodimers (Nolan *et al*, 1991). *In vivo* NFκB can be activated by diverse signals (see table 1.9)

which probably act by activating modifiers of IκB to release NFκB. Two kinases (PKC and haem-regulated kinase, HRI) could directly phosphorylate IκB *in vitro* whereas PKA could activate NFκB with much slower kinetics and independently of IκB phosphorylation (Ghosh and Baltimore, 1990), thus indicating that the transcription factor can be modulated by different pathways. In addition distinct forms of IκB (α=37kD and ß=40-48kD) were purified from human placenta cytosol indicating a further possible mechanism for activation by diverse agents (Zabel and Baeuerle, 1990). These studies have led to the proposal of a model for NFκB activation shown in figure 1.3.

Table 1.9 Activators of NFκB *in vivo* and *in vitro*.

Phorbol esters
PKA/PKC
Haem regulated kinase
HTLV *tat*
dsRNA
TNF/IL1
T cell mitogens

Some known activators of NFκB binding activity are shown. HTLV = human T lymphotropic virus. Examples of activation by these agents can be found in the following references: Nelsen *et al* (1988); Shirakawa and Mizel (1989); Ghosh and Baltimore (1990); Leung and Nabel (1988); Visvanathan and Goodbourn (1989); Lowenthal *et al* (1989); Osborn *et al* (1989) and Bohnlein *et al* (1988) respectively.

A number of species binding to the NFκB motif have been identified (see table 1.10). The confusion surrounding the identity of some of these factors has been resolved by cloning studies. PRDIIBF1, a 298kD protein isolated from a human expression library was unusual in having two pairs of C_2H_2 type zinc fingers separated by 1630 amino acids which both bound the human ßIFN positive response domain PRDII and H-2K^b κB sequences (Fan and Maniatis, 1990). This clone was not NFκB *per se* as indicated by the size and antibodies to it had a nuclear location (Fan and Maniatis, 1990). Other zinc finger proteins binding κB motifs obtained by screening with MBP-1 cDNA have also been isolated (Rustgi *et al*, 1990) as well as mouse (αA-CRYBP1, Nakamura *et al*, 1990) and rat (AGIE-BP1, Ron *et al*, 1991) members of this family. The p50 subunit of NFκB has been cloned from human (Kieran *et al*, 1990) and mouse (Ghosh *et al*, 1990). The human clone encoded a predicted 969 amino acid, 105kD protein of which only the N-terminal half bound the NFκB motif, presumably the 105kD protein is processed *in vivo* to 50kD. No known DNA binding motifs or transcriptional activation domains were present in this region, thus a novel DNA binding domain is involved with the *trans*-activation function possibly being carried out by p65. The N-terminal 366 amino acids were highly homologous to the avian reticuloendotheliosis virus v-*rel*, its proto-

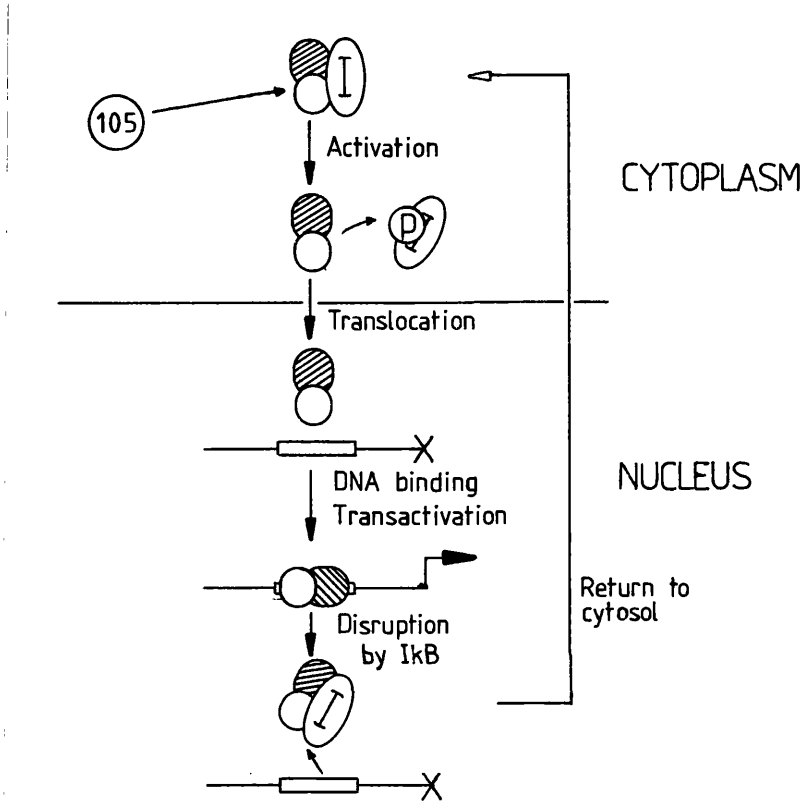


Figure 1.3 Model for Activation of NFκB Regulated Genes.
The p50 subunit (open circle) is derived from a 105kD precursor and associates with IκB (I) in conjunction with a p65 subunit (shaded). Phosphorylation of IκB releases the p50-p65 dimer which is then transported to the nucleus. There are a number of possibilities for multimer binding to the NFκB motif (shown as a rectangle). Here the p50-p65 dimer binds and activates transcription (in association with other factors). The small size of IκB (shown disproportionately here) suggests that it may be able to diffuse into the nucleus (via nuclear pores) disrupting the NFκB-DNA complex and allowing recycling to the cytoplasm. See text for references and further details.

oncogenic counterpart (c-*rel*), and the *Drosophila* maternal effect gene *dorsal* (Kieran *et al*, 1990; Ghosh *et al*, 1990). Ballard et al (1990) found 4 specific NFκB binding proteins of 50, 55, 75 and 85kD which were immunoprecipitable with anti-v-*rel* antibodies. p55 was probably the precursor to p50, whereas p75 could bind the NFκB site (with a lower affinity than p50) and could also dimerize with p50. These latter properties have now been attributed to p65 with the production of recombinant protein (Nolan *et al*, 1991; Ruben *et al*, 1991). Recombinant human c-*rel* was indistinguishable from p85 and may be the same as HIVEN86A (Ballard *et al*, 1990). Interestingly the different molecular weight forms are activated differentially in Jurkat cells following PMA treatment (Molitor *et al*, 1990).

Table 1.10 Factors Identified Which Bind the NFκB Motif.

1) MBP1/HIVEP1/PRDIIBF1
2) MBP2 (H2TF1)
3) KBP1
4) rat AGIE-BP1
5) mo αA-CRYBP1
6) hu p50/KBF1
7) mo p50
8) hu/mo p65 (p75)
9) hu c-*rel*/p85 (HIVEN86A)
10) p55

1-5 all contain zinc finger motifs which bind the NFκB site whereas 6-10 are *rel*-related and do not. All these proteins have now been cloned (assuming p55 is a precursor of p50). Factors in parentheses are thought to be the same as those shown or a precursor in the case of p75. Hu = human, mo = mouse. 1) Singh *et al* (1988); Maekawa *et al* (1989); Fan and Maniatis (1990); 2) and 3) Rustgi *et al* (1990); 4) angiotensinogen gene inducible enhancer binding protein 1, Ron *et al* (1991); 5) αA-cystallin binding protein 1, Nakamura *et al* (1990); 6) Kieran *et al* (1990); Bours *et al* (1990); Meyer *et al* (1991) 7) Ghosh *et al* (1990); 8) Ruben *et al* (1991); Nolan *et al* (1991); 9) and 10) Ballard *et al* (1990).

Specificity in NFκB activated genes may be brought about by differing distribution of members of the NFκB family, as well as inhibitors. In addition interaction with other transcription factors at a promoter are important, for example, TNFα and IL1 increased IL2-Rα expression in an immature T cell line (YT) in a manner dependent on the NFκB site, but not in Jurkat T leukaemic cells where NFκB was also activated (Freimuth *et al*, 1989). In addition the IL2-Rα and κ enhancer NFκB motifs bind NFκB with similar affinities but only the latter activates transcription from heterologous promoters (Cross *et al*, 1989). Hence IL2-Rα κB is cell specific and dependent on the presence of other factors binding the promoter. Similarly in Jurkat cells NFκB is required

for activation of the IL2 gene but is not sufficient with other factors such as NFAT-1 being required (Hoyos *et al*, 1989).

Thus NFκB is a molecule ideally suited to the rapid activation of gene expression, acting as both a messenger and transcriptional activator in many cell types where the context of the responding gene and the ability to bind various forms of NFκB provide the means for specific responses.

1.6 Aims of the Project.

In order to extend the studies carried out on human SAA this work aimed to delineate the structure of the human SAA genes by obtaining and characterizing genomic clones. In addition population studies using SAA probes and Southern hybridization would confirm the structure of the gene family and identify allelic variants. Comparison of the human genes and those of other species would thus enable possible evolutionary mechanisms to be proposed. Studies in other species have indicated that the SAA genes are differentially expressed (see section 1.2), the human genes could be used to investigate differences in responses to individual inflammatory stimuli. Thus an important aim was to address the question of the mechanisms involved in gene regulation during the inflammatory response with reference to potential *cis*-acting sequences. The availabilty of SAA genes would provide a model system for studying the interaction of cytokine pathways in activation of gene expression as well as the control of tissue specificity and inducibility.

In the following chapters the results of this research is presented with evidence for a human SAA gene family comprising four members. The transcriptional modulation of two of these genes by cytokines is also investigated and a model proposed for this process involving both NFκB-like and C/EBP-like factors.

CHAPTER 2

Materials and Methods.

2.1 Materials.

All reagents used were molecular biology grade and obtained from various sources. Enzymes, radionucleotides and other materials were obtained from the suppliers listed below.

Reagent.	Supplier.
ENZYMES.	
Restriction enzymes	Gibco Ltd., Uxbridge, UK.
	Northumberland Biologicals, Cramlington, Northumberland, UK
	Boehringer Mannheim, Lewes, Sussex, UK.
E. coli DNA polymerase I Klenow fragment	Pharmacia LKB Biotechnology Milton Keynes, UK.
T_4 DNA ligase	
T_4 polynucleotide kinase	
T_4 DNA polymerase	
Calf intestinal phosphatase	
RNase A, DNaseI	Sigma Chemical Co. Ltd., Poole, Dorset, UK.
Sequenase enzyme and reagents	United States Biochemicals, Cambridge Bioscience, Cambridge UK.
RADIOCHEMICALS.	
[α^{32}P]dCTP (3000 Ci mmol^{-1})	ICN Biochemicals Ltd., High Wycombe, Bucks., UK.
[γ^{32}P]ATP (3000 Ci mmol^{-1})	
[α^{35}S]dATP (100 Ci mmol^{-1})	Amersham International, Amersham, UK.
[^{14}C]chloramphenicol (57 mCi mmol^{-1})	
MEDIA.	
Yeast extract	Difco Laboratories, Detroit, MI, USA.
Bactotryptone	
Agar	
DMEM and additives	ICN Flow Laboratories, High Wycombe, Bucks., UK.
CYTOKINES.	
IL1ß (2x10^{8}Umg^{-1})	Genzyme Corporation, Boston, MA, USA.

IL6 (1-2x10^7Umg^{-1})	Amersham International, Amersham, UK.
IL6 (1x10^6Umg^{-1})	British Biotechnology Ltd., Oxford, UK.
TNFα (≥2x10^6Umg^{-1})	
Anti-IL1ß, anti-IL6 antibodies	National Institute of Biological Standards and Control.

PROTEASE INHIBITORS.

Aprotinin	Sigma Chemical Co. Ltd., Poole, Dorset, UK.
Iodoacetamide	
Leupeptin	
Pepstatin A	

SEQUENCING REAGENTS.

Polyethylene glycol and gel reagents	Sigma Chemical Co. Ltd., Poole, Dorset, UK.
SepPak C_{18} cartridges	Millipore Waters Associates, MA, USA.

OTHER.

Bluescript system	Stratagene Ltd, Cambridge Science Park, UK.
ϕX174 Hae III DNA markers	
λ HindIII DNA markers	Gibco Ltd., Uxbridge, UK.
Hybond-N, Hybond-C extra	Amersham International, Amersham, UK.
Random oligonucleotides pd$(N)_6$	Pharmacia LKB Biotechnology, Milton Keynes, UK.
dNTPs	
Acetyl CoA	
Allegro^{TM} hGH RIA	Biogenesis Ltd., Bournemouth UK.

2.1.2 General Buffers.

Recipes for general buffers and media follow, those used for specific reactions or procedures are given along with the protocols in section 2.2, others can be found by referring to Maniatis *et al* (1982). All solutions were prepared with double distilled water and sterilized by autoclaving or filtration through 0.45um membranes where necessary.

20xSSC:

3M NaCl; 0.3M Na_3citrate; pH 7.0.

20xSSPE:

3.6M NaCl; 0.2M $NaPO_4$; 0.02M EDTA.

100xDenhardt's solution:

2% (w/v) BSA; 2% (w/v) Ficoll;

2% (w/v) polyvinylpyrollidine.

10xURB:

0.33M Tris-OAc, pH 7.9; 0.66M KOAc; 0.10M MgOAc;

1mgml^{-1} BSA; 5mM DTT; 40mM spermidine.

50xTAE:

2M Tris; 50mM EDTA, pH 8.0; 1mM CH_3COOH.

10xTBES:

0.9M Tris; 0.89M boric acid; 25mM EDTA.

Polyacrylamide gel mix:

Appropriate volume of 40% 29:1 acrylamide : bis-acrylamide; 1xTBES; 0.13% AMPS; 0.08% TEMED.

PBS "A":

137mM NaCl; 2.7mM KCl; 1.5mM KH_2PO_4;

8.1mM Na_2HPO_4.

2.1.3 Broths and Media.

Superbroth:

1.2% (w/v) Bactotryptone; 2.4% (w/v) yeast extract; 0.5% (v/v) glycerol. After autoclaving K_2HPO_4 and KH_2PO_4 added to 7.2mM and 2.8mM final, respectively.

2xYT:

1.6% (w/v) bactotryptone; 1.0% (w/v) yeast extract; 85mM NaCl.

NZCYM:

1% (w/v) NZ amine; 0.1% (w/v) casamino acids; 0.5% (w/v) yeast extract; 85mM NaCl; 8mM $MgSO_4$.

SOB:

2% (w/v) Bactotryptone; 0.5% yeast extract;

10mM NaCl; 2.5mM KCl; 10mM $MgCl_2$; 10mM $MgSO_4$.

LB:

1% (w/v) Bactotryptone; 0.5% (w/v yeast extract);

170mM NaCl.

10x Hogness freezing medium:

36mM K_2HPO_4; 13mM KH_2PO_4; 20mM Na_3citrate;

10mM $MgSO_4$; 44% (v/v) glycerol.

Dulbecco's Modified Eagles Medium (DMEM):

DMEM; 10% (v/v) foetal calf serum; $2gl^{-1}$ $NaHCO_3$; $10Uml^{-1}$ penicillin; $10ugml^{-1}$ streptomycin; $25ugml^{-1}$ gentamycin; 0.5x non-essential amino acids; $0.294gl^{-1}$ L-glutamine.

2.1.4 Other.

Eschericia coli strains used:

TG1; DH5αF'; Q358; LE392

2.1.5 Oligonucleotides used in Band Shift Studies.

SAAκB wild-type

GATCCTGCAGGGACTTTCCCCAGG
GACGTCCCTGAAAGGGGTCCCTAG

SAAκB mutant

GATCCTGCA**CTC**ACTTTCCCAGG
GACGT**GAG**TGAAAGGGTCCCTAG

NF-IL6 site

AGCTTCGTCACATTGCACAATCTTAATA
AGCAGTGTAACGTGTTAGAATTATTCGA

IL6RE wild-type

AGCTTCCATAGGTTACACAACTGGGATA
AGGTATCCAATGTGTTGACCCTATTCGA

IL6RE mutant

AGCTTCCATAGG**C**TACAAA**C**CTGGGATA
AGGTATCC**G**ATGT**T**TGGACCCTATTCGA

2.2 METHODS.

2.2.1 DNA Mapping Studies.

2.2.1.1 Analysis of Genomic DNA.

5ug of human peripheral blood lymphocyte (HPBL) chromosomal DNA was digested with 20 units of the appropriate restriction enzyme in 40ul final volume under conditions described by the manufacturer. Digestion products were electrophoresed through 0.8% (w/v) agarose horizontal slab gels containing $0.5ugml^{-1}$ ethidium bromide in 1 x TAE at $1Vcm^{-1}$ for 20hr (unless otherwise stated). DNA was transferred to Hybond-N nylon membranes by capillary transfer using the method of Southern (1975) and the Amersham Hybond protocols. Briefly, depurination was carried out in 0.25M HCl for 20min, followed by denaturation in 1.5M NaCl, 0.5M NaOH for 45min and neutralization in 1.0M Tris, pH7.0, 1.5M NaCl for 60min. Gels were blotted overnight in 20xSSC and DNA cross-linked to the dried membranes using a u.v. Stratalinker (Stratagene).

Membranes were prehybridized in 5xSSC, 5xDenhardt's solution, 0.5% SDS, $100ugml^{-1}$ denatured sonicated salmon sperm DNA at 65°C for 2-4hr before the addition of 10^5-10^6cpm ml^{-1} [^{32}P]DNA probe (labelled to >10^8cpm ug^{-1} see section 2.2.2.2).

Following hybridization at 65°C for 20hr membranes were washed with increasing stringency from 2xSSC, 0.1% SDS at room temperature to 0.5xSSC, 0.1% SDS at 65°C until background counts were removed. Membranes were exposed to Kodak XAR X-ray film with intensifying screens at -70°C.

2.2.1.2 Isolation and 32P-Labelling of DNA Fragments.

2.2.1.2.1 Isolation of Cloned DNA Fragments.

20-30ug of plasmid DNA was digested with appropriate restriction enzyme(s) under standard conditions. Digestion products were loaded onto wide slots on 0.7-1.0% (w/v) agarose gels, electrophoresed and run onto Whatman DE81 filter papers pretreated in 2.5M NaCl. DNA was eluted in 1.5M NaCl in TE at 37°C for 2hr and filtered through GF/C (Whatman), phenol/chloroform extracted, ethanol precipitated and resuspended in TE.

2.2.1.2.2 ^{32}P-Labelling of DNA with Random Primers.

Random hexanucleotide primers annealed to 65-100ng DNA were extended using the Klenow fragment of *E.coli* DNA polymerase I and [α^{32}P]dCTP according to Feinberg and Vogelstein (1983). After incubation at 37°C for 1hr unincorporated dNTPs were separated from labelled probe on a Sepharose CL-6B spin column and specific activity determined in a Cerenkov counter.

2.2.1.2.3 Klenow labelling of recessed 3' ends.

1ug of DNA containing 3' recessed ends produced by restriction endonuclease cleavage (e.g. HindIII) were filled in using 1 unit of Klenow fragment in nick translation buffer (Rigby *et al*, 1977) with at least one of the 4 dNTPs labelled (e.g. [α^{32}P]dCTP). The reaction was carried out at 37°C for 30min and terminated by the addition of 0.1 vols 3M NaOAc and 2.5 vols absolute ethanol.

Where gel purification was required the precipitated fragments were resuspended and electrophoresed through 0.4% polyacrylamide vertical slab gels in 1 x TBE. After autoradiography of the wet gel, the required bands were cut out and electrophoresed onto DE81 and purified as described in section 2.2.1.2.1 or electroeluted into a high salt (3M NaOAc) trap, diluted in H_2O and ethanol precipitated.

10x Nick Buffer: 500mM Tris-Cl, pH 7.2; 100mM $MgSO_4$;
1mM DTT; 500ugml^{-1} BSA.

2.2.1.2.4 Labelling with T4 DNA Polymerase.

The combined 3'-5' exonuclease and 5'-3' polymerase activities of T4 DNA polymerase were used to end label restricted DNA fragments in an exchange (replacement) reaction. 1ug of DNA was mixed with 5ul of [α^{32}P]dCTP (3000Cimmol^{-1})and 1ul of T4 DNA polymerase in 1xURB buffer in a total volume of 25ul. After 30min. at 37°C the reaction was terminated by ethanol precipitation and the labelled fragment gel purified as described above or purified on a Sephadex G-50 column.

2.2.1.2.5 5'-end Labelling with T4 Polynucleotide Kinase.

Labelling was carried out essentially as described by Maxam and Gilbert (1980)

for protruding 5' termini. 50ng of oligonucleotide or 1ug of restricted DNA lacking the 5' phosphate group was incubated at 37°C for 30min in 10ul kinase buffer containing 2ul [γ^{32}P]ATP and 3 units of T4 polynucleotide kinase. Labelled DNA was purified from polyacrylamide gels or by centrifugation through Sepharose CL-6B as described above.

10x kinase buffer: 500mM Tris-Cl, pH 7.6; 100mM $MgCl_2$;
50mM DTT; 500ugml^{-1} BSA;
1mM spermidine.

2.2.1.3 Screening of Genomic Libraries.

2.2.1.3.1 λ DNA Libraries.

The procedure followed has been described by Benton and Davis (1977). 10^6 plaques were plated on indicator bacteria (e.g. *E.coli* LE392) in 150mm petri dishes containing NZCYM agar. After overnight incubation nitrocellulose replicas were made in duplicate and treated in 0.5M NaOH, 1.5M NaCl for 1min, neutralized in 1.5M NaCl, 0.5M Tris, pH8.0 for 5min and rinsed in 2xSSPE. DNA was fixed to the filters by baking in a vacuum oven for 2hr.

Filters were prehybridized overnight at 42°C in 50% formamide, 5xDenhardt's, 5xSSPE, 0.1% SDS, 100ugml^{-1} denatured sonicated salmon sperm DNA, 1ugml^{-1} *E.coli* DNA and 0.1ugml^{-1} λ DNA. 10^5-10^6cpmml^{-1} [^{32}P]DNA labelled as described in section 2.2.1.2.2 was added to the hybridization mixture and incubation continued at 42°C for a further 20hr. Non-specific activity was removed by washing sequentially in 2xSSC, 1xSSC and 0.5xSSC containing 0.1% SDS until no background activity was detected. Positive hybridizing clones were identified by autoradiography. Phage from agar plugs picked from the positive region were eluted in SM, a dilution plated on 85mm dishes and the screening repeated until single isolated plaques could be picked. Preparation of DNA from bacteriophage recombinants is described below.

2.2.1.3.2 Cosmid Libraries.

2 x 10^5 recombinant bacteria were plated on 20x20cm Hybond-N membranes on LB-agar containing appropriate antibiotics and grown at 37°C until each colony was 0.1-0.2mm diameter. Replicas were made using the procedure of Hanahan and Meselson (1983). The master filter was placed onto a pad of dampened Whatman 3MM paper, a second prewetted filter aligned on top and the two pressed together using even pressure applied with a glass plate. The replica filter was removed to a fresh antibiotic plate and 2 further replicas taken (the third after returning the master filter to 37°C for 2hr). Master and replica plates were grown at 37°C until 1mm diameter colonies formed (about 6hr for the replicas). For immediate screening master plates were stored at 4°C otherwise immediately after the replicas were taken the master filters were transferred to antibiotic plates containing Hogness freezing medium and grown for 1-2hr. A sandwich was then made by placing a second filter prewetted in freezing medium on top of the master, this was then stored at -70°C between 3MM paper sealed in plastic.

Replica filters were treated by placing sequentially on a pad of 3MM soaked in 10% SDS for 3min., 0.5M NaOH, 1.5M NaCl for 7min., 1.5M NaCl, 0.5M Tris-Cl, pH7.2 for 2 x 3min. and finally washed in 2 x SSPE for 10min. Membranes were fixed and hybridized as described in section 2.2.1.1 with the inclusion of 10ugml^{-1} denatured plasmid DNA. Probe inserts were isolated by electrophoresing twice through agarose to remove plasmid sequences.

Following identification of positive hybridizing clones master filters were returned to agar plates and grown at 37^{o}C for 1h. Positive colonies were scraped off into 200ul of antibiotic medium and serial dilutions plated, further screening was carried out until individual positive colonies could be picked.

2.2.1.4 DNA Preparations.

2.2.1.4.1 λ DNA Preparations.

Recombinant λ DNA was isolated by a modification of the infection method described by Maniatis *et al* (1982). 2x10^{8} pfu (plaque forming units) were added to 10^{10} bacterial host cells (e.g. *E.coli* Q358). After 15 min incubation at 37^{o}C 250ml of 2xYT was added and shaking continued for 5-6hr until cells were lysed. 800ul of chloroform was added and shaking continued for 15 min to complete lysis. Lysed cultures were spun in a Sorvall GSA rotor at 8000rpm for 10 min at room temperature. Phage were precipitated by stirring the supernatant overnight with 25g polyethyleneglycol (PEG, 6000), 15g NaCl, 15mg RNase, 1mg DNase at 4^{o}C and respun in the GSA rotor at 8000rpm for 30 min at 4^{o}C. The pellet was resuspended in 2ml SM and extracted with an equal volume of chloroform, vortexed for 30secs and spun at 1500g in a Sorvall bench top at 4^{o}C. The resulting supernatant was spun in a Beckman SW41 rotor for 2hr at 2500rpm at 4^{o}C. The pellet was resuspended in 600ul of SM, RNase added to 100ugml^{-1} and incubated at 37^{o}C for 30min. EDTA was added to 20mM, SDS to 0.1%, proteinase K to 0.2mgml^{-1} and incubated at 65^{o}C for 30min. Following phenol/chloroform extraction the DNA was precipitated, spooled out and resuspended in TE.

SM: 0.1M NaCl; 50mM Tris-Cl, pH7.5; 8mM $MgSO_4$;
0.04% gelatin.

2.2.1.4.2 Cosmid DNA Preparations.

i) Large scale preparation.

A single colony harbouring the recombinant cosmid/plasmid was picked into 10ml LB broth containing appropriate antibiotics and shaken at 37^{o}C until cloudy (3-4hr). This culture was added to 100ml antibiotic-broth and grown for a further 3-4hr when it was added to 400ml of superbroth with antibiotic and grown up overnight at 37^{o}C with shaking.

Bacteria were pelleted in a Sorvall GS3 rotor at 6500rpm for 5min at 4^{o}C. Cosmid/plasmid DNA was then isolated using the alkali-lysis method of Ish-Horowicz and Burke (1981). Closed circular DNA was then purified by centrifugation through a

caesium chloride-ethidium bromide gradient in a Beckman Ti70.1 rotor at 54000rpm for 20hr. The DNA band was isolated, ethidium removed by butanol extraction and DNA precipitated in ethanol to yield 5mg.

ii) Small scale preparation.

Bacteria were grown overnight in 2ml cultures and 1.5ml pelleted at 12000rpm in eppendorf tubes. Plasmids were isolated by the alkaline lysis method and after treatment with solution 3 the supernatant was ethanol precipitated. After pelleting and drying the DNA was resuspended in 40ul H_2O and 4ul core restriction buffer, 1ul of 10mgml^{-1} RNaseA was added and incubated at 37^{o}C for 2hr. This yielded 15-20ug of DNA of sufficient purity for restriction endonuclease analysis. Phenol/chloroform extraction following treatment with solution 3 was required for use in dideoxy sequencing reactions (see below). For cosmid clones (rather than plasmid) 3ml of culture was pelleted per clone and a phenol/chloroform step included to isolate DNA of reasonable purity and yield.

2.2.1.5 Characterization of Recombinant DNA Clones.

2.2.1.5.1 Restriction Endonuclease Analysis of Recombinant DNA.

2-3ug of bacteriophage, cosmid or 1ug plasmid DNA purified as described above was incubated with appropriate restriction endonucleases under standard conditions (generally 3-5 units incubated at 37^{o}C for 1-2hr in buffers supplied). For multiple digests, endonucleases were incubated concurrently with the DNA in URB restriction buffer (Tartof and Hobbs, 1988). All enzymes were found to cleave in this buffer although the activity of KpnI and SmaI was variable. Restriction fragments were separated on agarose of the required concentration at 3Vcm^{-1} for 2-4hr and DNA visualized by u.v. flourescence of intercalated EtBr.

2.2.1.5.2 Southern Clone Blotting.

Gels were treated and blotted onto Hybond-N membranes as described for genomic DNA in section 2.2.1.1 with the exceptions that transfer was for 15min with 2 or 3 blots per gel being made. Hybridization conditions were the same as genomic blots using 10^{5}cpmml^{-1} of [^{32}P] labelled probe. Filters were washed sequentially in solutions containing 2xSSC, 1xSSC and 0.5xSSC with 0.1% SDS at room temperature for 30min each. A high stringency 0.5xSSC, 0.1% SDS wash at 65^{o}C was then carried out for 10-30min if required. Hybridizing fragments were identified by autoradiography.

2.2.1.5.3 Subcloning of DNA Fragments.

To further characterize regions of interest DNA was cut with restriction endonucleases and fragments isolated as described in section 2.2.1.2.1. Plasmid DNA (e.g. Bluescript KS) was cut in the multicloning region to produce ends complementary to those of the insert. 250ng of insert DNA was incubated with 25ng of cut vector in a 10ul reaction containing 50mM Tris-Cl, pH7.8, 10mM $MgCl_2$, 20mM DTT, 2mM ATP and 5 units of T4 DNA ligase at 15^{o}C for 20hr. The ligation mixture was added directly to 100ul of competent *E.coli* DH5αF', incubated on ice for 1hr and heat shocked at 44^{o}C for

90secs. Following a further 15min on ice 800ul of SOB containing 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose was added and the cells grown at 37°C for 30min. Cells were pelleted in a microfuge for 1min, resuspended in 200ul SOB and plated on LB agar containing $2x10^{-3}$% X-GAL and IPTG plus appropriate antibiotics. Insertional inactivation of the ß-galactosidase gene in the vector allowed selection of recombinants due to inability to metabolize X-GAL giving white colonies.

2.2.1.5.4 Preparation of Competent *E.coli.*

Competent cells were prepared to give an efficiency of 10^8 transformed cells per ug of DNA using the rubidium chloride method exactly as described by Glover (1985). Incubation times in RF1 were 15min on ice for all strains used and in RF2 were 15min on ice for TG1 or 30min for DH5αF'.

2.2.1.6 DNA Sequence Analysis.

2.2.1.6.1 Preparation of Single Stranded DNA.

DNA was sequenced using the dideoxy chain termination method of Sanger *et al*, (1977) and modified T7 DNA polymerase (Tabor and Richardson, 1987). Single stranded DNA was prepared by cloning into M13mp18 and mp19 vectors with growth in a suitable host according to Messing (1983). Inserts ligated into an M13 vector under conditions described in section 2.2.1.5.3 were used to transform 100ul of cells carrying the F pilus (e.g. TG1, DH5αF'). Following incubation on ice for 1hr and heat shock at 42°C for 2min (TG1) or 44°C for 90secs (DH5αF') the cell suspension was added to 3ml of top agar (0.7% w/v) containing 200ul exponential culture (0.4-0.6 $O.D._{600}$), 10ul 2% IPTG, 50ul 2% X-GAL and poured on a 2xYT plate. After incubation overnight at 37°C colourless plaques were picked into 2ml 2xYT medium with 50ul exponential *E.coli.* Cultures were grown for 6hr at 37°C with vigourous shaking and bacteria pelleted in a microfuge for 10min. Phage were precipitated from the medium by incubation with 0.2vols of 20%PEG, 2.5M NaCl at room temperature for 15min. This was then spun for 10min in the microfuge and all liquid removed from the pellet which was then resuspended in 100ul 1.1M NH_4OAc, pH7.0, phenol/chloroform extracted and ethanol precipitated.

2.2.1.6.2 DNA Sequencing Reactions.

Typically 1ug of DNA was used in a sequencing reaction with [α^{35}S]dATP label as described in the Sequenase protocols. For sequencing close to the primer a 1:10 or 1:15 dilution of the labelling mix was used to increase the relative concentrations of the ddNTPs in the termination reaction. In addition extension and termination reactions were kept to a maximum of 3 mins each at room temperature and 37°C respectively. For extended reactions the concentration of [α^{35}S]dATP was doubled with extension and termination at the described temperatures for 5min each. Pausing of the polymerase on templates caused by secondary structure effects were resolved using dITP as described in the sequencing manual. Templates in plasmid vectors were denatured using the method of Hattori and Sakaki (1986) and 2-3ug used in sequencing reactions with suitable primers.

2.2.1.6.3. Denaturing Gel Electrophoresis.

Sequencing reaction products were separated by heating samples to 75-80°C for 2min and then loading 1ul onto 6-8% denaturing polyacrylamide gels which had been prerun for 10-15min. Gels wre 40 x 20 x 0.04cm, 19:1 acrylamide:bis-acrylamide containing 7M urea run in 1 x TBES at $30Vcm^{-1}$ maintaining a surface temperature of 50°C. For resolution over a greater number of nucleotides 0.4 to 0.8 mm wedge gels were run. Buffer gradient gels were prepared by taking up 8ml of 0.5 x TBES/acrylamide/urea mix followed by 12ml of 2.5 x TBE/acrylamide/urea mix and 4 air bubbles introduced. This was then poured between the plates and topped up with the 0.5 x mix. Following polymerization gradient gels were run at $30vcm^{-1}$ with 0.5 x TBES in the top chamber and 1 x TBES in the bottom chamber for a maximum of 3hr without prerunning.

After electrophoresis gels were fixed in 10% acetic acid, 10% methanol for 15min, dried at 80°C on a vacuum drier and exposed to Kodak XAR film for 8-35hr.

Standard denaturing gels:

Appropriate volume of 40% 19:1 acrylamide:bis-acrylamide: deionised, filtered and <1 week old; 50% (w/v) urea; 1xTBES; 0.07% AMPS; 0.07% TEMED.

Gradient gel mixes.

0.5 TBE gel mix: 6% 19:1 acrylamide:bis-acrylamide; 46% (w/v) urea; 0.5xTBES.

5.0 TBE gel mix: 6% 19:1 acrylamide:bis-acrylamide; 46% (w/v) urea; 0.5xTBES.

AMPS and TEMED added to 0.07% before forming gradient.

2.2.1.6.4 Preparation of Oligonucleotides.

Sequencing oligonucleotides were synthesized on a Milligen 7500 DNA synthesizer. The support material from the dried columns was incubated in 1.5ml of $NH_{(l)}$ at 55-65°C overnight to hydrolyze the oligonucleotide from the silica resin and deblock. Following cooling on ice the supernatant was dried under vacuum, resuspended in water and passed down a SepPak C_{18} column. After phenol/chloroform extraction and ethanol precipitation 0.5pmol were used to prime 1ug of template DNA.

2.2.1.6.5 Analysis of Sequence Data.

Primary sequence data were recorded and collated using the Microgenie program (Beckman). Further alignments, database comparisons *etc.* were carried out using the University of Wisconsin Genetics Computer Group (GCG) Package. For multiple sequence comparisons the program Clustal was used (Higgins and Sharp, 1988). Derived amino acid sequences were used for phylogenetic comparisons with the PROTPARS program of the phylogeny inference package PHYLIP developed by J. Felsenstein (Felsenstein, 1990).

2.2.2 Gene Expression Studies.

2.2.2.1 Tissue Culture.

All procedures were carried out in a class II flow cabinet. The human hepatoma cell line HepG2 (mycoplasma free) was maintained in Dulbecco's modified Eagles medium (DMEM) containing 10% (v/v) heat inactivated foetal calf serum (FCS). Cells were incubated in a humidified incubator at 37°C with 5% CO_2 in T_{75} flasks and passaged 1:3 at confluence (every 4-7 days). After aspiration of the medium 1ml of versene trypsin was added and flasks incubated at 37°C for 2-3 min. The detached cells were collected and spun through 10ml of fresh medium at 6000rpm for 8min at 22°C in an MSE Mistral 3000 and 1-2x10^6 cells plated in T_{75} flasks with 8ml of medium.

2.2.2.2 Transfection Studies.

2.2.2.2.1 Plasmid Constructions.

Various lengths of SAA 5' flanking region were cloned upstream of the bacterial chloramphenicol acetyl transferase (CAT) gene in the vector pTKCAT (Miksicek *et al*, 1987) with the TK promoter removed. Strategies used for cloning are described below (section 5.1).

2.2.2.2.2 Transient Transfection by the Calcium Phosphate Technique.

The calcium phosphate technique of gene transfer first described by Graham and van der Eb (1973) was used to transfect exogenous DNA into tissue culture cells. For transfection of HepG2 cells in 6 well plates, generally 8ug of test plasmid DNA was cotransfected with 3ug of the human growth hormone gene under the mouse metallothionein (MMT) promoter (plasmid pXGH5, as internal control). 200ul of DNA in 0.1 x TE, pH8.0 containing 0.25M $CaCl_2$ was added dropwise with agitation to 200ul of freshly prepared 2 x HBS, pH7.12±0.05. The calcium phosphate/DNA precipitate was allowed to form by standing at room temperature for 30min. The precipitate was then added dropwise with swirling to 5x10^5 HepG2 cells which had been fed 3-4hr previously with DMEM plus 2ugml^{-1} fungizone. After 4-24hr incubation at 37°C/5% CO_2 cells were checked for the appearance of a fine precipitate washed with 2ml of serum free medium and refed with complete medium. Growth hormone samples were taken 24hr after transfection by collecting 200ul of medium and using 100ul in the Allegro HGH RIA to quantitate HGH secreted.

2.2.2.2.3 Treatment of Cells with Cytokines.

For transcriptional studies using the transient transfection system cells were treated 24hrs after transfection. Cells were treated with the following concentrations of cytokines unless indicated otherwise:- IL1ß at 1Uml^{-1}, IL6 at 100Uml^{-1} and TNFα at 100Uml^{-1} added directly to the culture medium. Cells were harvested at various times following treatment.

2.2.2.2.4 Assay for Chloramphenicol Acetyl Transferase Activity.

CAT production directed by constructs transfected into mammalian cells was assayed using the method of Gorman *et al* (1982). Cells in 6 well plates were washed

with 2ml of warm PBS"A", aspirated and 130ul of TTE added at room temperature. Following 3 cycles of freeze-thawing the cell lysate was transferred to eppendorf tubes with the addition of 40ul of TTE used to wash the wells and left on ice for 10 min. Following incubation at 60°C for 10min to inactivate acetylases the extracts were spun for 10min at 25000g at room temperature. The supernatant was used immediately in CAT assays or the extracts were snap frozen and stored at -70°C.

25-50ul of cell extract was incubated with 8ul ^{14}C-chloramphenicol, 5ul of 80% glycerol and 20ul of 4mM acetyl Co-A for 20-120min at 37°C as indicated. Reactions were terminated by the addition of 1ml of ice cold ethyl acetate, vortexed for 20sec and spun at 25000g for 10min. 950ul of the upper organic phase was removed and dried under vacuum. The residue was resuspended in 15ul of ethyl acetate and spotted onto silica gel coated thin layer chromatography plates. Chromatography was carried out in 95:5 chloroform:methanol equilibrated tanks at room temperature for 1hr and products visualized by overnight exposure to X-ray film. Acetylated and non-acetylated products were cut out and activity quantitated in a beta scintillation counter.

Results were normalized for transfection efficiency by measuring hGH secreted into the medium as a result of cotransfection with pXGH5. HGH was assayed using the Allegro immunoassay kit (Biogenesis Ltd) and protocols provided. The formation of avidin:biotin-Ab(1):HGH:^{125}I-Ab(2) complexes was quantitated in a gamma counter.

CAT activity was calculated as:-

$$\frac{\text{acetylated counts}}{\text{unacetylated counts}} \times 100 \times \frac{\text{mean HGH}}{\text{HGH}}$$

10xHBS: 8.18% NaCl; 5.94% Hepes; 0.20% Na_2HPO_4; diluted to 2xHBS, pH7.12±0.05 with NaOH.

TTE: 250mM Tris-Cl, pH 7.5; 0.5% Triton X-100; 5mM EDTA.

2.2.2.2.5 Data Analysis for Transcriptional Studies.

Data from CAT assays were pooled and analyzed using the analysis of variance program ANOVA of Minitab 6.1. The three data columns consisted of "experiment number" (1, 2, etc.), "+/- cytokine" (*i.e.* 1=no cytokine, 2=+ cytokine), and "value" (log[normalized % chloramphenicol converted]). Using the output from ANOVA mean induction ratio and 95% confidence intervals were calculated from:

$$(x_1 - x_2) \pm (\text{SE DIFF} \times M)$$

where x_1 and x_2 = means of the induced and uninduced values respectively, M = multiplier from standard t tables,

$$\text{SE DIFF} = \frac{\text{mean SE} \times Y}{N}$$

where Y = number of variables in column 2 (*i.e.* 2), N = number of variables in column 1 (*i.e.* number of experiments). Final values were obtained by taking exponentials of results from the first equation.

2.2.2.3 DNA Binding Studies.

2.2.2.3.1 Preparation of Cellular Extracts.

Nuclear extracts were prepared from HepG2 cells following treatment with agonists using the method of Dignam *et al* (1983). All procedures were carried out at 4°C. For each extract $5x10^7$ cells were trypsinized, spun twice through ice-cold PBS"A" and resuspended in 5 packed cell volumes (PCV) of buffer "A" containing protease inhibitors. Cells were pelleted at 2000rpm for 15min at 4°C in a MSE Mistral 3000 and resuspended in 2 PCV of buffer "A". Cells were dounce homogenized using a type "A" pestle for 50 strokes to achieve >90% cell lysis as seen microscopically. The cell suspension was spun at 25000g in an Eppendorf centrifuge for 20min and the crude nuclei resuspended in 1ml of buffer "C" containing protease inhibitors. This was Dounce homogenized for 10 strokes and stirred at 4°C for 30min. The suspension was respun at 25000g for 20min and the supernatant dialyzed against >50 volumes of buffer "D" overnight with one buffer change. Dialyzed material was spun at 25000g for 20min, snap frozen and stored at -70°C. S100 fractions were prepared by centrifuging the supernatant from the first 25000g spin in 1x buffer "B" in a Beckman SW55 rotor at 32000rpm/4°C for 70min the supernatant of which was snap frozen.

Protein concentrations in nuclear extracts were determined using the Sigma BCA-1 assay and bovine serum albumin standards.

Buffer A: 10mM Hepes, pH 7.9; 1.5mM $MgCl_2$; 10mM KCl; 0.5mM PMSF; 0.5mM DTT.

Buffer B: 300mM Hepes, pH 7.9; 1.4M KCl; 30mM $MgCl_2$.

Buffer C: 20mM Hepes, pH 7.9; 420mM NaCl; 1.5mM $MgCl_2$; 0.2mM EDTA; 25% glycerol; 0.5mM PMSF; 0.5mM DTT.

Buffer D: 20mM Hepes, pH 7.9; 100mM KCl; 0.2mM EDTA; 20% glycerol; 0.5mM PMSF, 0.5mM DTT.

Protease inhibitors were added to buffers to give $1ugml^{-1}$ each of pepstatin A, iodoacetamide, leupeptin and aprotinin.

2.2.2.3.2. Electrophoretic Mobility Shift Assays.

Analysis of protein-DNA complex formation was carried out as described by Sen and Baltimore (1986). Typically reactions contained 10-15ug of total nuclear proteins preincubated for 5-10min at room temperature in a 20ul reaction volume with 1xbinding reaction buffer and $300ugml^{-1}$ poly(dI-dC) as non-specific competitor. 10^5cpm of gel purified ^{32}P end labelled DNA fragment (prepared as described in sections 2.2.1.2.3-5) was added to the reaction and incubaton carried out at room temperature for 15-30min. For competition studies the reaction was incubated for 15min at room temperature with

specific unlabelled competitor DNA before the addition of probe. Reactions were terminated by the addition of 2ul of type II DNA loading buffer and bound/unbound fragments separated by electrophoresis through a 4% polyacrylamide bandshift gel at $8Vcm^{-1}$ for 2-3hr with buffer circulation. Bands were visualized by autoradiography after drying down.

10X binding reaction buffer:

40% glycerol; 10mM EDTA; 50mM DTT; 10mM Tris-Cl, pH 7.5; $1mgml^{-1}$ BSA; 10mM to 1M NaCl.

10X band shift buffer (BSB):

33mM NaOAc; 67mM Tris-Cl, pH 7.5; 10mM EDTA; 0.5mM ßME.

Band shift gel:

Appropriate volume of 40% 29:1 acrylamide : bis-acrylamide; 1xBSB; 0.2mM DTT; 0.2% AMPS; 0.06% TEMED.

2.2.2.3.3 DNA Footprinting Studies.

For interference footprinting 100ng of *SAA2* 265bp fragment was end-labelled and gel isolated as described above to give a specific activity of $>10^8$dpm ug^{-1}. Base specific chemistry was then carried out on 10^7dpm of labelled fragment as described by Maxam and Gilbert (1980). For methylation interference, labelled fragment was resuspended in 200ul of $G\text{-}G_0$ and incubated with 1ul of dimethyl sulphate (DMS) for 30sec at room temperature before addition of 50ul of DMS stop solution. For C + T specific reactions, 30ul of hydrazine was added to labelled fragment in 20ul of water and incubated for 8min at room temperature before addition of 200ul of CT stop. Fragments were EtOH precipitated washed in 70% EtOH and resuspended in 10ul of water. Bandshift reactions were carried out under conditions described above using 10-fold higher protein concentrations in 150ul final volume. Bound and unbound products were separated on bandshift gels and the wet gels exposed to film for 2hr. Shifted and non-shifted fragments were cut out and electroeluted. Samples were resuspended in 10% piperidine and incubated at 90°C for 30-40min. After washing to remove piperidine, samples were resuspended in 90% formamide and approximately 10^5dpm run per lane on sequencing gels.

$G\text{-}G_0$: 50mM Na cacodylate; 1mM EDTA.

DMS Stop: 1.5M NaOAc; 1M ßME; $100ugml^{-1}$ poly-d(I-C).

CT Stop: 0.3M NaOAc; 0.1mM EDTA; $25ugml^{-1}$ poly-d(I-C).

CHAPTER 3.

Structural Analysis of the Human Serum Amyloid A Gene Family.

3.1 Introduction.

Previous studies on human SAA had shown that six major protein isoforms were detectable in the sera of acute phase individuals (Bausserman *et al*, 1980) however the basis for the production of these proteins was unknown. For example the isoforms could be products of individual gene loci, alleles of one or more loci, or variants of protein processing. The later cloning of distinct cDNA sequences (Sipe *et al*, 1985; 1986; Kluve-Beckerman *et al*, 1986a) suggested that there was more than one SAA gene in humans. This was compatible with the then contemporary findings of a gene family for SAA in the mouse (Lowell *et al*, 1986a, Yamamoto *et al*, 1987). When work on this project commenced a genomic clone λSAAg9 had been characterized, this was predicted to be an allele of one of the cDNAs (pSAA82, Kluve-Beckerman *et al*, 1986a) however the overall structure of the human SAA genes was unclear. Thus the aim of this work was to determine the complexity of the human SAA gene family by cloning and population studies. Thus it would be possible to answer questions concerning evolution and patterns of expression of the genes and provide a basis for determinig possible structural alterations within the genes which maybe linked to deposition of amyloid fibrils as occurs in other amyloid syndromes (*e.g.* familial amyloidotic polyneuropathy, Wallace *et al*, 1988). Cloning of the SAA genes would therefore be a precursor to approaching these and other problems.

3.2 Results.

3.2.1 Southern Analysis and Detection of Polymorphisms.

Initial steps in the characterization of the human SAA gene family involved Southern analysis of genomic DNA from unrelated individuals using the human SAA cDNA pA1 (Sipe *et al*, 1985) as probe. The clone pA1 carries an SAA1 insert which contains both a phylogenetically conserved region in the 5' PstI fragment (pA1a) and diverged/human specific sequences in the 3' PstI fragment (pA1b). Restriction digestion products were separated by agarose gel electrophoresis and blotted onto Hybond-N membranes as described (section 2.2.1.1). Following hybridization of filters with ^{32}P-labelled pA1a or pA1b, filters were washed at low stringency (2 x 40min in 2xSSC, 0.1%SDS, followed by 40min in 0.5xSSC, 0.1%SDS at room temperature). The restriction digests studied were BglI, BglII, EcoRI, HindIII, NcoI, PstI and TaqI. Representative autoradiographs are shown in figure 3.1.

Hybridization of HindIII blots with pA1a or pA1b produced three constant bands of 10.5, 3.6 and 1.3kb with a restriction fragment length polymorphism (RFLP) producing bands of 4.6 and 3.0kb. The 10.5 and 3.6kb bands showed least homology to pA1 and were undetectable at higher stringency. RFLPs were also detected for BglI fragments probed with pA1a: in addition to the constant bands at 5.8, 2.7 and 2.2kb, 3.25,

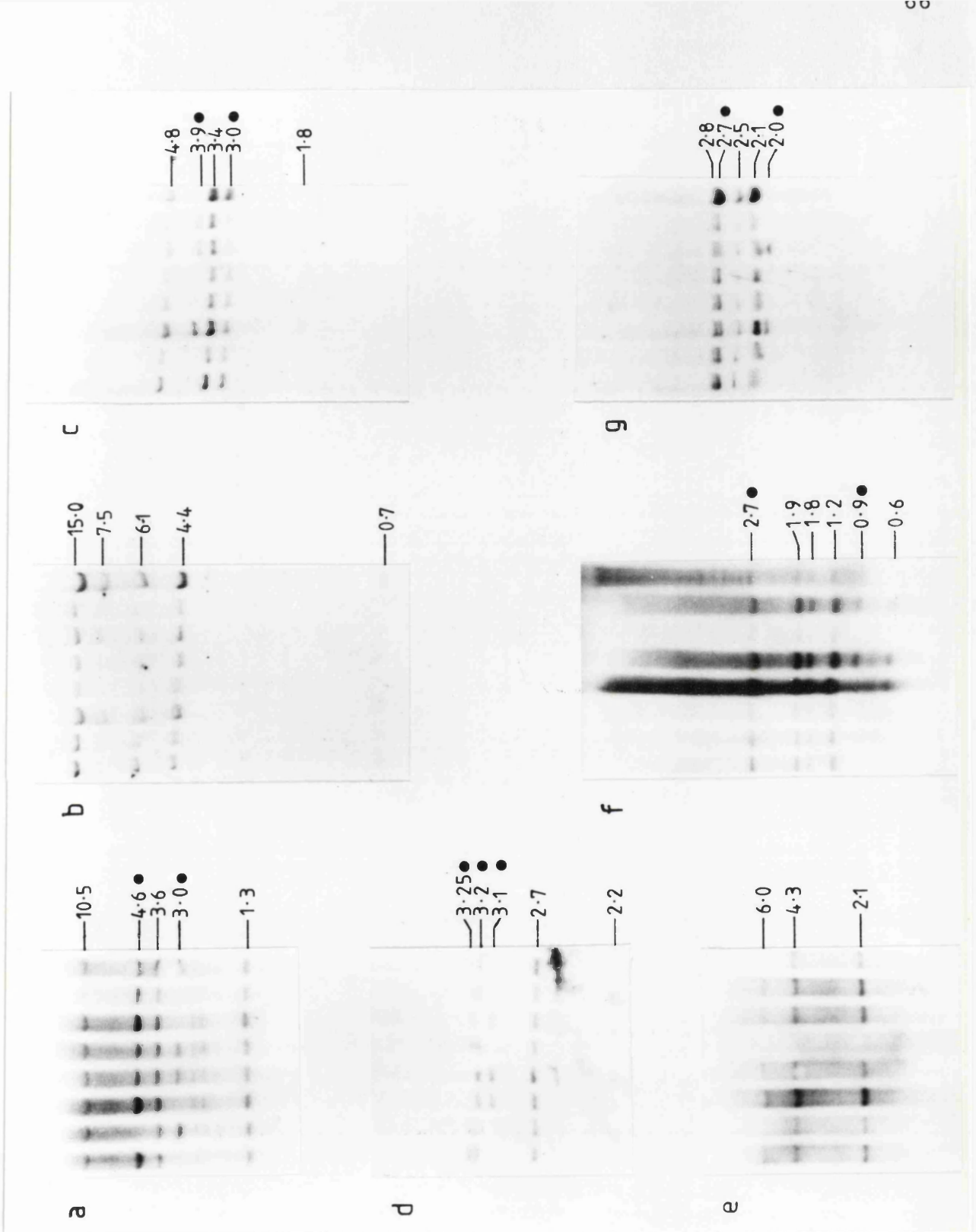


Figure 3.1 Southern Analysis of Human Genomic DNA with SAA Probes.
5ug of human genomic DNA was cleaved separated on agarose, blotted onto Hybond-N and probed with pA1a or pA1b. a) HindIII, pA1a; b) BglII, pA1b; c) NcoI, pA1b; d) BglI, pA1a; e) TaqI, pA1b; f) PstI, pA1a; g) PstI, pA1b. Fragment sizes in kb are shown to the right with polymorphic fragments denoted by black circles.

3.2 and 3.1kb polymorphic fragments were observed. It was necessary to run 1.4% agarose gels at $1Vcm^{-1}$ for 65hr to resolve the BglI polymorphic bands due to their similar molecular weights.. The 5.8 and 2.2kb fragments showed least homology to pA1a. NcoI digestion also created polymorphic fragments of 3.5 and 2.7kb detected with pA1b, in addition constant bands were present at 4.8, 3.3 and 0.7kb. PstI digests produced a number of bands with polymorphic fragments at 2.7 and 2.0kb with pA1b. BglII and TaqI digests were also carried out on the same population of approximately 30 unrelated Caucasians but RFLPs were not detected although polymorphisms were found by other investigators using SAA probes (Sack *et al*, 1988; Steinkasserer *at al*, 1990; Shohat *et al*, 1990).

Digestion of human DNA with BglII produced identical hybridizing bands at 15, 7.5, 6.1 and 4.4kb with both pA1a and pA1b and a 700bp fragment exclusive to pA1b. EcoRI digests contained >20, 12.0, 11.0 and 1.4kb pA1a and pA1b hybridizing fragments, with a 5.5kb band recognized by pA1a only and 15.0 and 1.7kb bands appearing with pA1b only.

Examination of the Southern blotting data revealed the presence of about 3 or 4 constant bands with each of the digests with additional bands created by polymorphic sites. This suggested that the human SAA gene family comprised 3 or 4 members.

3.2.2 Family and Population Studies of SAA Alleles.

The polymorphisms detected for HindIII and BglI were investigated further to determine i) whether they were allelic and thus inherited in a Mendelian fashion,

and ii) the polymorphism information content (PIC) to assess the applicability of these RFLPs as markers for genetic mapping studies.

In an analysis of 3 families comprising 68 members the HindIII 4.6kb (allele 1) and 3.0kb (allele 2) bands were found to be inherited in a Mendelian fashion. The PIC was calculated using the equation of Botstein *et al* (1980) which for 2 alleles at a single locus was simplified to

$$1-(p_1^2+p_2^2)-2p_1^2p_2^2$$

where "p" is the frequency of alleles 1 and 2. Among 54 unrelated Caucasians the frequencies of genotypes 1,1, 1,2 and 2,2 were 32, 19 and 3 respectively. Hence p_1=83/108 and p_2=25/108 or 0.769 and 0.231. Substituting into the above equation gives a PIC value of 0.293. Thus this would be a reasonably informative marker in mapping studies. A PIC of >0.5 is considered highly informative for a 2 allele system however the low frequency of allele 2 in this population reduced the PIC.

The 3 variable BglI bands were studied in 5 families with 82 members and demonstrated that they were allelic (an example of inheritance in one branch of a family is shown in figure 3.2). For 3 alleles PIC was calculated using

$$1-(p_1^2+p_2^2+p_3^2)-2p_1^2p_2^2+2p_1^2p_3^2+2p_2^2p_3^2.$$

The frequencies for alleles 1, 2 and 3 were 0.264, 0.170 and 0.566 respectively among 53 unrelated individuals, giving a PIC of 0.514. A maximum score of 0.6 is possible for the

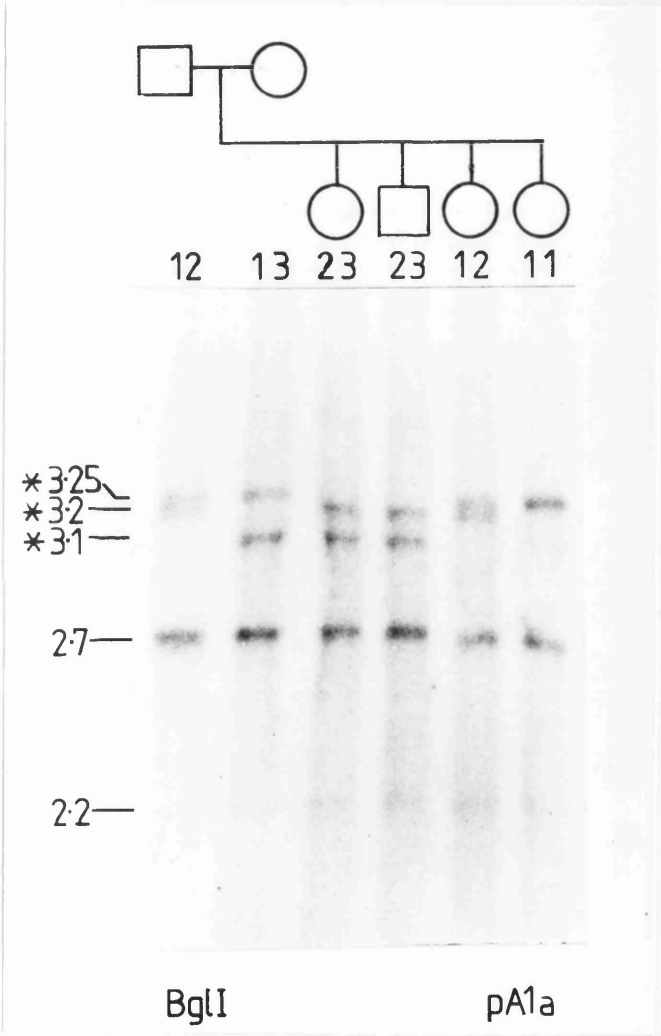


Figure 3.2 Mendelian Inheritance of BglI Alleles.
The inheritance of the BglI alleles 1, 2 and 3 is shown in one branch of a family. Sizes of the hybridizing fragments are shown to the left in kb with the polymorphic bands indicated by an asterisk. The genotypes for each of the individuals is shown at the top of each lane.

even distribution of 3 alleles in a population. Hence the SAA BglI polymorphism would be reasonably informative.

The genotypes for 14 unrelated Caucasians were determined for the BglI, HindIII, NcoI and PstI RFLPs. These data, presented in table 3.1 provide some evidence that the NcoI and PstI polymorphisms may be linked with NcoI allele 1 (3.9kb) only occurring when the Pst allele 2 (2.0kb) was present. Also in 3 out of 4 cases where HindIII allele 2 was present the aforementioned NcoI and PstI alleles (1 and 2, respectively) were also present. In one case (individual 9) this was not true being heterozygous for the HindIII marker but homozygous for NcoI allele 2 and PstI allele 1. From this limited study there did not seem to be any association between the BglI genotype and those for the other markers.

Table 3.1 SAA Genotypes in a Caucasian Population.

Individual	BglI	HindIII	NcoI	PstI
1	3,3	1,1	2,2	1,1
2	1,3	1,1	2,2	1,1
3	3,3	1,1	2,2	1,1
4	3,3	1,1	2,2	1,1
5	1,3	1,1	2,2	1,1
6	1,3	1,1	2,2	1,1
7	-	1,2	1,2	1,2
8	1,3	1,1	2,2	1,1
9	1,2	1,2	2,2	1,1
10	1,3	1,2	1,2	1,2
11	3,3	1,2	1,2	1,2
12	3,3	1,1	2,2	1,1
13	1,2	-	-	1,1
14	2,2	-	-	2,2

Genotypes are shown for the 4 RFLPs detected in these studies for 14 unrelated Caucasians. The highest molecular weight allele was designated 1 in each digest (see figure 3.1). Dashes indicate that no result was obtained.

3.2.3 Isolation and Characterization of Human SAA Genomic Clones.

3.2.3.1 Isolation of 4 Novel λ Genomic Clones.

Previous screening of human genomic libraries led to the isolation of a number of clones including SAAg9 (Woo *et al*, 1987), GSAA1 and GSAA3 (Sack, 1983). To further delineate the SAA locus a human genomic library in λEMBL3 was screened using the SAA cDNA pA10 (Sipe *et al*, 1986). From the positive plaques obtained 4 were chosen for further analysis (λ1.1, 1.2, 1.3 and 2.2). These clones were mapped using a series of

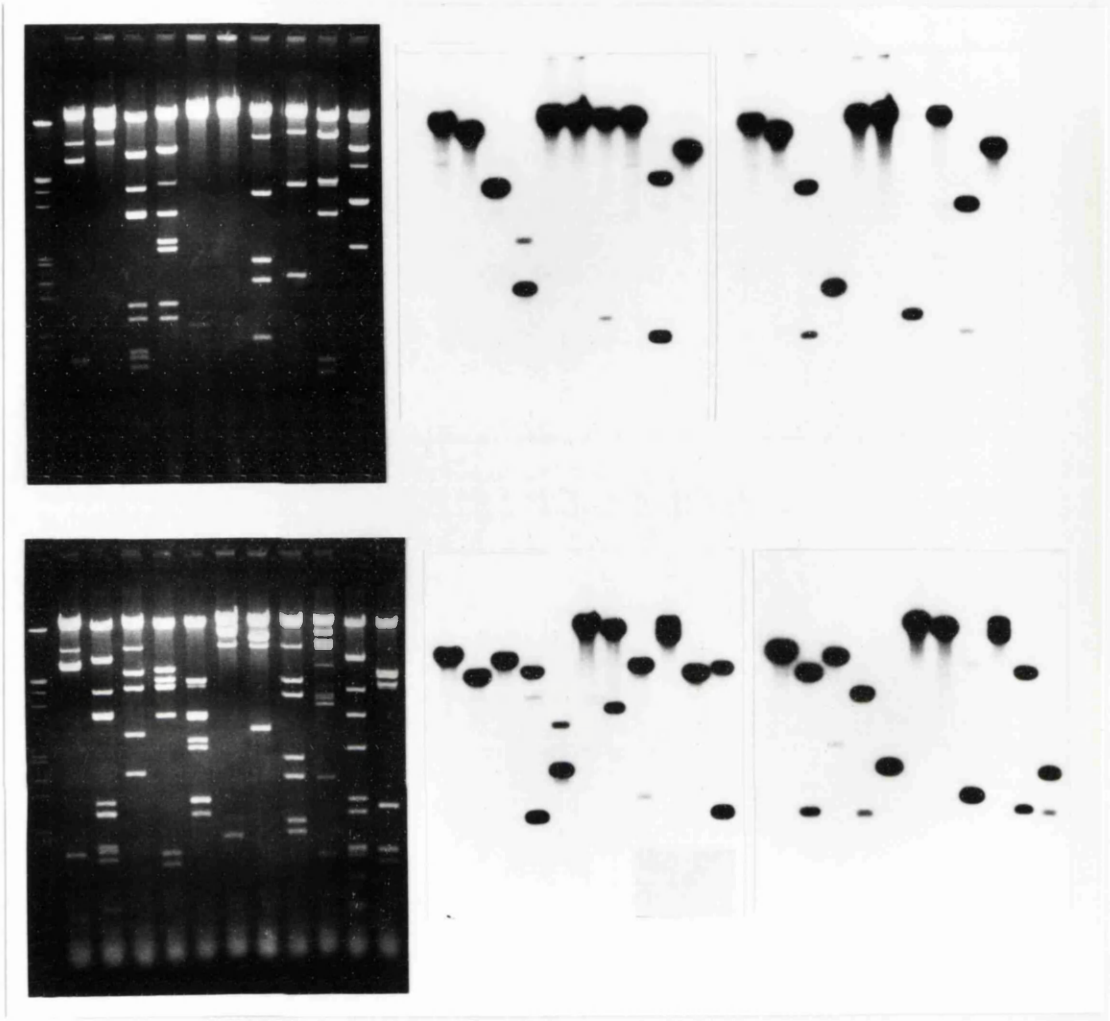
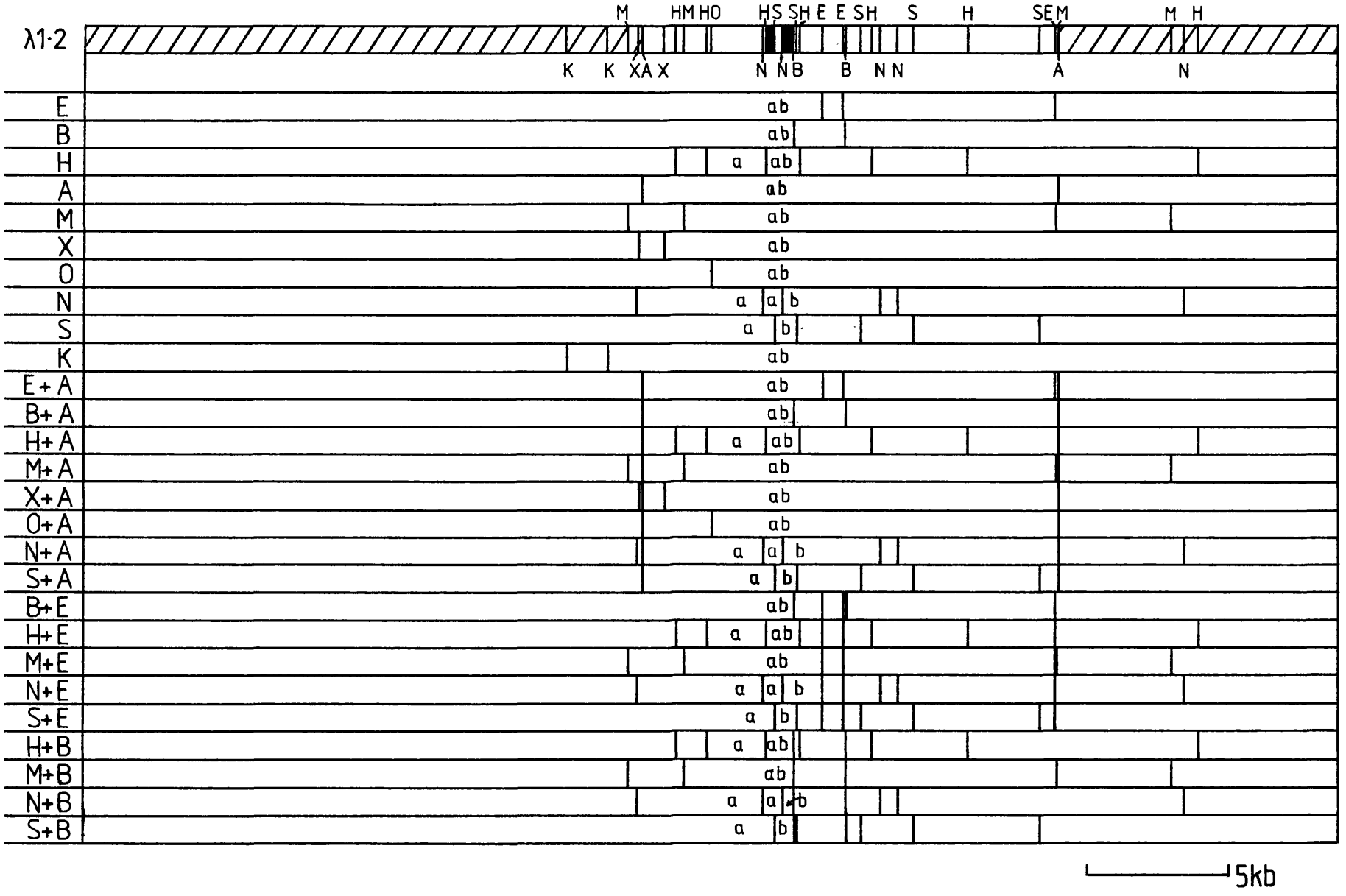


Figure 3.3 Restriction Mapping of Lambda 1.2.
Lambda 1.2 DNA was digested with various restriction enzymes and visualized by ethidium fluorescence after electrophoresis through agarose (left). Corresponding clone blots were hybridized with pA1a (centre) or pA1b (right). EcoRI+HindIII lambda DNA was used as molecular weight markers (first lane of each gel) with lambda 1.2 digests (top, left to right) EcoRI, SalI, BglII, HindIII, XbaI, ShoI, SstI, BamHI, NcoI, SmaI, (bottom) SalI+EcoRI, SalI+BglII, SalI+BamHI, SalI+NcoI, SalI+HindIII, SalI+XbaI, SalI+XhoI, SalI+SstI, SalI+SmaI, EcoRI+BglII, EcoRI+NcoI.

Figure 3.4 (next page) **Construction of Lambda 1.2 Restriction Map.**
Examples are shown of restriction digests and resolution of fragments into a physical map for lambda 1.2. Fragments hybridizing to pA1a and pA1b are shown and were delimited to the solid regions shown at the top. The arms of the lambda vector are shaded. Key: E=EcoRI, B=BamHI, H=HindIII, A=SalI, M=SmaI, X=XbaI, O=XhoI, N=NcoI, S=SstI, K=KpnI.


λ1·2
M HM HO HS SH E E SH S H SE M M H
K K XA X N N B B N N A N
E
B
H
A
M
X
O
N
S
K
E + A
B + A
H + A
M + A
X + A
O + A
N + A
S + A
B + E
H + E
M + E
N + E
S + E
H + B
M + B
N + B
S + B
a ab
5kb

single and double digests. Restriction fragments containing SAA coding sequences were identified by hybridization to pA1a and pA1b on Southern clone blots. Restriction fragments visualized by fluorescence of intercalated ethidium bromide under uv and the corresponding Southern blot hybridized to pA1a and pA1b are shown for digests of λ1.2 in figure 3.3. Examination of such gels shows that, for example, digestion of λ1.2 with HindIII produces 8 fragments ranging from 1.0kb to 21kb. Of these the 1.3kb band hybridizes strongly to both pA1a and pA1b whereas a 2.2kb band hybridizes weakly to pA1a only. A HindIII/EcoRI double digest does not affect the hybridization pattern indicating that there are no EcoRI sites within the SAA gene HindIII fragments. However both NcoI and SstI cleave in this area. Using such reasoning the maps for the 4 λ clones were elucidated. An example of this is shown for the mapping of λ1.2 in figure 3.4.

The maps for λ1.1, 1.2 and 1.3 all had similar pA1 hybridizing fragments (by molecular weight and hybridization intensity), including a 1.3kb HindIII pA1 hybridizing fragment, a 1.0kb fragment hybridizing to pA1b and a 750bp pA1b-hybridizing NcoI fragment. λ2.2 also had a similar 750bp fragment produced by NcoI digestion but lacked other similarities in the hybridizing region. To gain more detailed information in this area the 2.2kb HindIII fragment from λ1.2, the 1.3kb HindIII fragment from λ1.1, 1.2 and 1.3 as well as the 2.8kb PstI fragment from λ2.2 were subcloned into the appropriate sites in pUC18. The restriction maps for these subclones (pUC1-5) are shown in figure 3.5. pUC2, 3 and 4 were indistinguishable on the basis of the restriction mapping carried out. The pUC1 clone hybridized weakly to pA1a and showed no similarity to the other subclones. pUC2-4 pUC5 hybridized both pA1a and pA1b but the latter was dissimilar in terms of restriction fragment sizes.

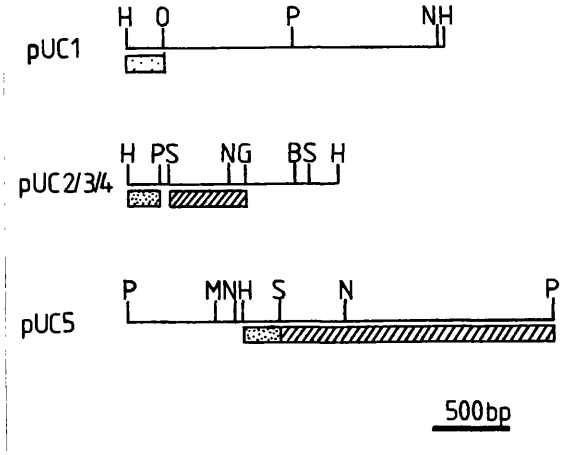


Figure 3.5 Restriction maps of the pUC1-5 Inserts.
Mapping data for the 5 inserts is shown with fragments hybridizing to pA1a (stippled) and pA1b (hatched) as indicated. The HindIII-XhoI fragment of pUC1 only hybridized weakly to pA1a and no pA1b hybridization was detected. pUC2-4 were indistinguishable on the basis of these maps. Abbreviations as figure 3.4.

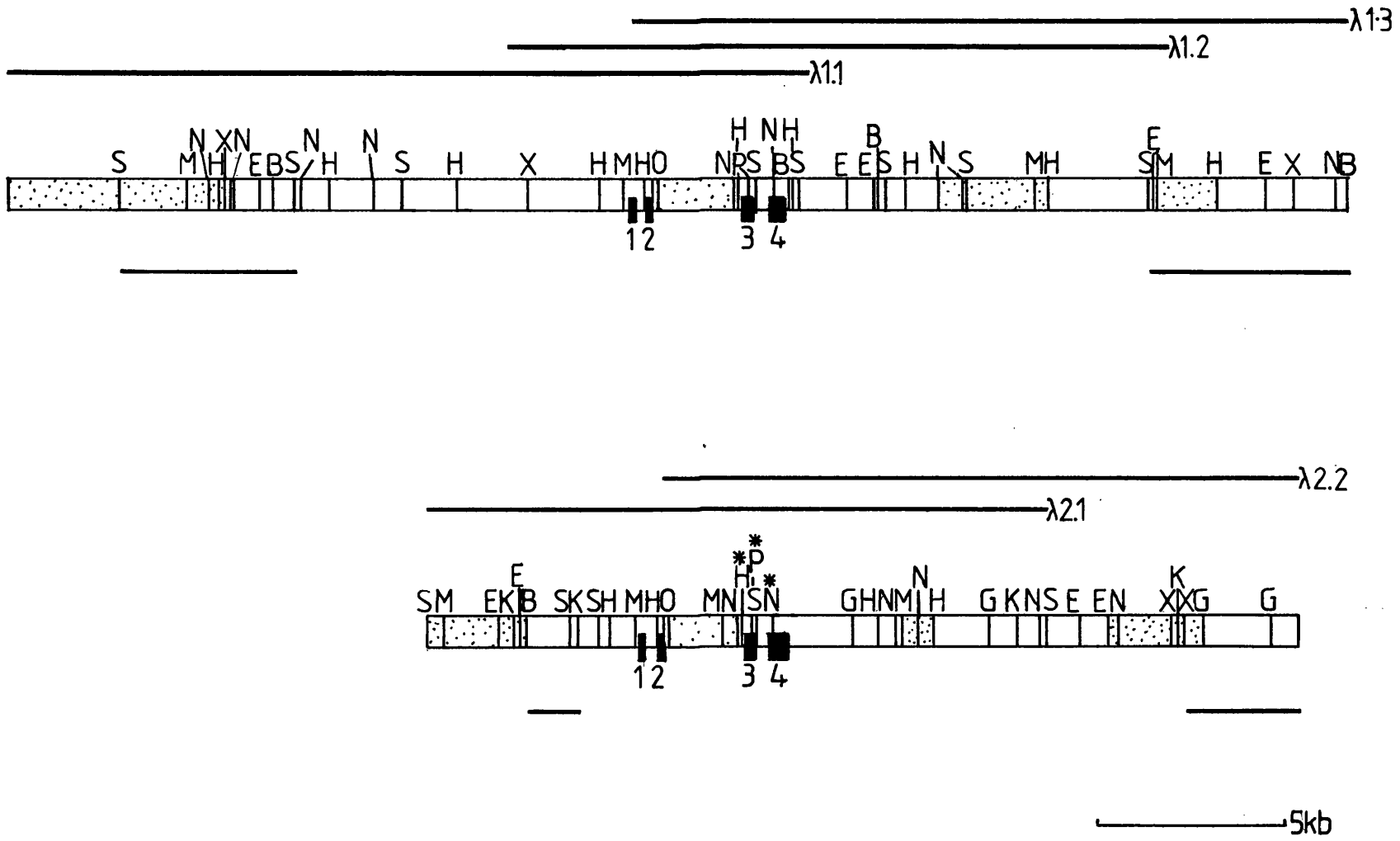


Figure 3.6 Restriction Map of Contiguous λ Clones at SAA Loci.
Maps are shown for the 2 gene loci characterized with the positions of exons determined from sequence analysis. * denotes sites which were polymorphic between λ2.1 and λ2.2 (only the polymorphic PstI site is shown). Shaded regions represent fragments containing repetitive DNA hybridizing to a total human genomic DNA probe. Underlined fragments were subcloned for chromosome walking (see figure 3.9). Abbreviations for restriction sites as figure 3.4.

The identity between these regions of λ1.1, 1.2 and 1.3 and sequence analysis (see below) as well as further similarities in the restriction patterns of λ1.2 and 1.3 in non-coding regions, suggested that these clones were contiguous (see fig.3.6). Although several similarities existed with the previously characterized λSAAg9 (hereafter called λ2.1) these clones had no homology in the regions flanking the coding region and thus were from a distinct locus. λ2.2 also had a number of restriction site differences from λ2.1 in the SAA homologous region. However there was striking similarity between the remainder of the restriction map and the 3' non-coding region of λ2.1. These two clones were therefore represented overlapping chromosomal regions with a number of polymorphic sites existing between them.

3.2.3.2 DNA Sequence Analysis of Human SAA Genomic Clones.

3.2.3.2.1 Sequencing Strategy.

Restriction fragments from pUC1-5 and from λ clones were subcloned into M13mp18 and mp19 and Bluescript KSM13-. Inserts were sequenced using the strategy shown in figure 3.7 by the dideoxy chain termination method (section 2.2.1.6). The results of sequence analysis are shown in figure 3.8 compared to the known sequence for *SAA2ß* encoded by λ2.1 (Woo *et al*, 1987).

3.2.3.2.2 λ1.2 Encodes ApoSAA1ß.

λ1.2 shows strong homology to λ2.1 (fig. 3.8). λ1.2 contains a 4 exon gene, slightly longer than *SAA2ß* (3640bp from the putative cap site to the poly(A) addition signal). Exon/intron boundaries were predicted using the GT-AG rule for donor and acceptor mRNA splice sites and correlate with the known cDNA sequences. Alignment of the 5' and 3' splice sites for introns 1, 2 and 3 with the consensus from an extensive compilation of genes (Mount, 1982) is shown in table 3.2. The 5' splice sites conform well with the AG/GTAAGT consensus (/ = splice site). The 3' splice sites all have a polypyrimidine tract, a non-conserved position, a pyrimidine and the absolutely conserved AG dinucleotide (Mount, 1982).

The derived amino acid sequence of λ1.2 is identical to apoSAA1 with Ala^{52} and Val^{57} and hence encodes the ß isoform as defined by Parmelee *et al* (1982). This gene has not previously been cloned although the cDNA pA1 encodes an allele apoSAA1α with Val^{52} and Ala^{57}. The *SAA1ß* gene has nine amino acid differences in 7 codons of exon 4 compared to *SAA2ß*, one of which is synonomous the remainder giving rise to 6 amino acid differences at residues 60, 68, 69, 71, 84 and 90. Sequence analysis of equivalent regions from λ1.1 and λ1.3 showed identity with that from λ1.2 between nucleotides 3607 to 3841 and 4514 to 4794 (i.e. the regions sequenced).

The nucleotide sequence of *SAA1ß* shows 90% sequence conservation with *SAA2ß* from 450bp upstream of the transcriptional start site extending to the 3'-untranslated region of exon 4. Outside this region no homology is evident with the exception of sequence elements upstream of the 5' limit of homology (see below: sequence of *SAA1* 5' region). A 300bp *Alu* repeat insertion (*SAA1AluB*, nt 3115 to 3382)

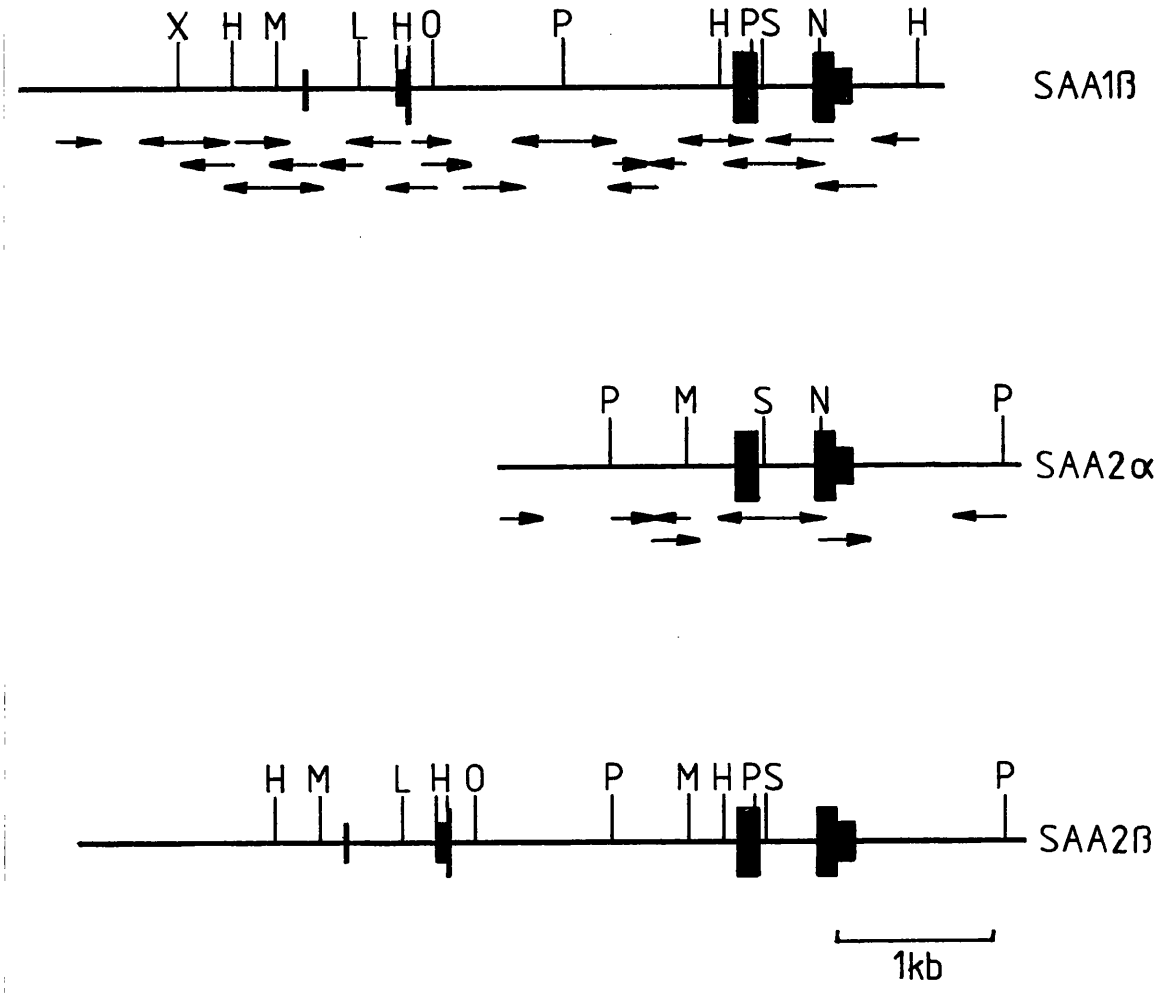


Figure 3.7 Sequencing Strategy for SAA Genomic Clones.
Clones are shown aligned with *SAA2β* with restriction sites used in subcloning (key as fig. 3.4) for sequencing. Arrows show the extent and direction of the sequencing carried out. Large and narrow boxes denote translated and untranslated regions of exons.

in intron 2 of *SAA1β* interrupts this homology. A further *Alu* repeat is also present (*SAA1AluA*, nt 2668 to 2947) which is also present in *SAA2* intron 2 in an analogous position. The *Alu* sequences present in the human SAA genes are discussed in section 3.2.7.1.

Table 3.2 Alignment of 3' and 5' Splice Sites for SAA Sequences.

	5' splice	A_{64}	G_{73}	I	G_{100}	U_{100}	A_{62}	A_{68}	G_{84}	U_{63}
SAA1β	Intron 1	A	G	I	G	U	G	A	G	G
	Intron 2	U	G	I	G	U	A	A	G	G
	Intron 3	A	G	I	G	U	A	A	C	U
SAA2α	Intron 3	A	G	I	G	U	A	A	C	U

	3' splice	(U/C)*n*	N	(U/C)	A_{100}	G_{100}	I	G
SAA1β	Intron 1	$(U/C)_{11}$	U	C	A	G	I	C
	Intron 2	$(U/C)_{15}$	C	C	A	G	I	G
	Intron 3	$(U/C)_{23}$	A	C	A	G	I	C
SAA2α	Intron 2	$(U/C)_{15}$	C	C	A	G	I	G
	Intron 3	$(U/C)_{23}$	A	C	A	G	I	C

The 3' and 5' splice sites for the genes sequenced in this study are shown aligned. The consensus sequences in the top rows have the percentage occurrence of each nucleotide in a compilation of splice sites (Mount, 1982) shown as a subscript. The subscripts adjacent to the (U/C)s of the 3' splice sites show the number of nucleotides covered by these regions which were 93, 100, 72, 100 and 68% (U/C)-rich for *SAA1* introns 1-3 and *SAA2* introns 2 and 3 respectively.

3.2.3.2.3 λ2.2 Encodes ApoSAA2α.

Partial sequence analysis of λ2.2 was carried out, the results are shown in figure 3.8. The genomic insert terminated at the 5' end of intron 2 but the sequence of exons 3 and 4 showed identity with *SAA2ß* with the exception of a T to C change at codon 54 which is silent in the reading frame (GCT to GCC remaining as alanine). This polymorphism was recognized during restriction mapping due to the loss of the PstI site present in exon 3 of *SAA2ß*. A further difference occurs in exon 4 at codon 71 where the CGT of *SAA2ß* encoding arginine is CAT encoding histidine, hence the predicted protein product is apoSAA2α. The G/A difference creates an NcoI polymorphism: present in *SAA2α* (CCATGG) but lacking in *SAA2ß* (CCGTGG), this was evident during restriction mapping and population Southern analysis. *SAA2α* has previously been characterized at the cDNA (pA10, pSAA82, pAS_8, Sipe *et al*, 1986; Kluve-Beckerman *et al*, 1986; Steinkasserer *et al*, 1990) and protein levels (Strachan *et al*, 1989; Kluve-Beckerman *et al*, 1988). A third single nucleotide change creating a restriction site polymorphism was detected towards the 3' end of intron 2 by HindIII (ATGCTT in *SAA2α* compared to AAGCTT in *SAA2ß*) creating 4.6kb and 3.0kb alleles studied by Southern analysis. Two of the restriction site differences (NcoI and PstI) were caused by a C to T transition at a

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SAA1b  TCTAGACTGAGGGTGAAGGAGCTTCCAGGGCACACATGAGACATGGCAGGGCTAGGCTGCTAGTTTTATTTTGTTTTGTTTTAGACACAGGGTCTTGCTC 100
SAA2b  ---AATCTGACCCTGTTGATGTTCTCATGAGAGA---GTGATCTGA-ATGCCCC--CTGA-ACCCCTCCGTGATAATACAGCAGACCAAGAGCTCTCCCA

SAA1b  TGTTAACCAGGCTGGAGTGCAGTGGCGTGATTATAGCTCA-CTGCAGCCTTGACCTCCTGGGTCTCCCACAATCCTTCCGCTTCAGCCTCTTGAGTAG-C 198
SAA2b  CCCTTCCCTGCCTGGA-TGCTG-GGCACGTCCCCAGCTGGGCTGCCTATTTAACGCACCACA-CTCTCATTCTCCCAA-GGTGGGGCTCCAGGACTAGGC

SAA1b  TGGGACTGCAGGT-GCACACTACCACACCCGGTCCATTTATTTTTATATTTCGTAGAGACAAGATCTTACAGTTTTGCACAG--AGTG-ATCTTAAACTC 294
SAA2b  TGGGGCAGCAGAAAGTCCCCCTCTCTACATTGTCCTTGGCTCAGGAGCCAACTTAGAAA-AAGCATTTCCAAATTGGCTAAGCCAGCGGAGCAGAGATTT

SAA1b  TTGACCCCAAGTGATCCTCCTGCCTT----GGCCTCCAA--AAGCATTGGGATTATAGGAGTGA-GCCACTGTGCTGGACC-TAGTCTGTCAGCTTTGAA 386
SAA2b  TCTGTGCTGAGAAATATCAGGACATCCAGAGGGGTGGAAGGAGGCTTCCAGGGCACACATGAGATGTGGCAGGGGTAGGCTGTCCGTTTTAAAGCTTAAA

SAA1b  GCTTTAGATATGAACTCAGAGGGACTTCATTTCAGAGGCATCTGCCATGTGGCCCAGCAGAGCCCATCCTGAGGAAATGACTGGTAGAGTCAGGAGCTGG 486
SAA2b  GCTTTAGACATGAACTCACAGGGACTTCAGT-CAGGGTCATCTGCCATGTGGCCCAGCAGGGCCCATCCTGAGGAAATGACCGGTATAGTCAGGAGCTGG

SAA1b  CTTCAAAGCTGCCCTCACTTCACACCTTCCAGCAGCCCAGGTGCCGCCATCACGGGGCTCCCACTCTCAACTCCGCAGCCTCAGCCCCCTCAATGCTGAG 586
SAA2b  CTGAAGAGCTGCCCTCACTCCACACCTTCCAGCAGCCCAGGTGCCGCCATCACGGGGCTCCCACTGGCATCTCTGCAGCTGCACTTCCCCCAATGCTGAG

SAA1b  GAGCAGAGCTGGTCTCCTGCCCTGACAGCTGCCA-GGCACA---------TCTTGTTCCCTCAGGTTGCACAACTGGGATAAATGACCCGGGATGAAGAA 676
SAA2b  GAGCAGAGCTGATCTAGCACCCTGTCCATTGCCAAGGCACAGCAAACCTCTCTTGTTCCCATAGGTTACACAACTGGGATAAATGACCCGGGATGAAGAA

SAA1b  ACCACTGGCATCCAGGAACTTGTCTTAGACCGTTTTGTAGGGGAAATGACCTGCAGGGACTTTCCCCAGGGACCACATCCAGCTTTTCTTCCCTCCCAAG 776
SAA2b  ACCACCGGCATCCAGGAACTTGTCTTAGACCAGTTTGTAGGGGAAATGACCTGCAGGGACTTTCCCCAGGGACCACATCCAGCTTTTCTTCCCTCCCAAG

SAA1b  AAACCAGCAGGGAAGGCTCAGTATAAATAGCAGCCACCGCTCCCTGGCAGGC**AGGGACCCGCAGCTCAGCTACAGCACAGATCAG**GTGAGGAGCACACCA 876
SAA2b  AGACCAGCA----AGGCTCACTATAAATAGCAGCCACCTCTCCCTGGCAG**ACAGGGACCCGCAGCTCAGCTACAGCACAGATCAG**GTGAGGAGCACAC-A

SAA1b  AGGAGTGATTTTTAAAACTTACTCTGTTTTCTCTTTCCCAACAAGATTATCATTTCCTTTAAAAAAAATAGTTATCCTGGGGCATACAGCCATACCATTC 976
SAA2b  AGGAGTGATTTTTAAAACTTACTCTGTTTTCTCTTTCCCAACAAGATTATCATTTCCTTTAAAAAAAATAGTTATCCTGGGGCATACAGCCATACCATTC

SAA1b  TGAAGGTGTCTTATCTCCTCTGATCTAGAGAGGTAAGCAGGGTCGGGCCTGGTA-GTACTTGGATGGGAGAACACCTGGGAATACCAGGTGCTAAAGGCT 1075
SAA2b  TGAAGGTGTCTTATCTCCTCTGATCTAG----GTAAGCAGGGTCGGGCCTGGTAAGTACTTGGATGGGAGAACACCTGGGAATACCAG-TGCTAAAGGCT

SAA1b  TTAAGAATAAAAAATAATGATCCTGCTTTGTGTTTATGGGATGTTGAGTTCTGTG-CGGGG--CA-GAGGG-AACACACGGTAAATGCGTTATGGGGAAT 1170
SAA2b  TTAAGAATAAAAAATAATGATCCTGCTTTGTTTATCCCC-ATGTTGAGTTCTGTGTCGGTGGTCATGAGGGTAACACACGGTAAATGCGTTATGGGGAAT

SAA1b  TAT-AGGCTACTTGAGGGAGTGA-CAGTCTGGTGGTAACTCCTGCCTTCCTCCATCAGTGCCACGTTGGC-TCCTCTTATGCAGTCAGGCTTCAGGGCTG 1267
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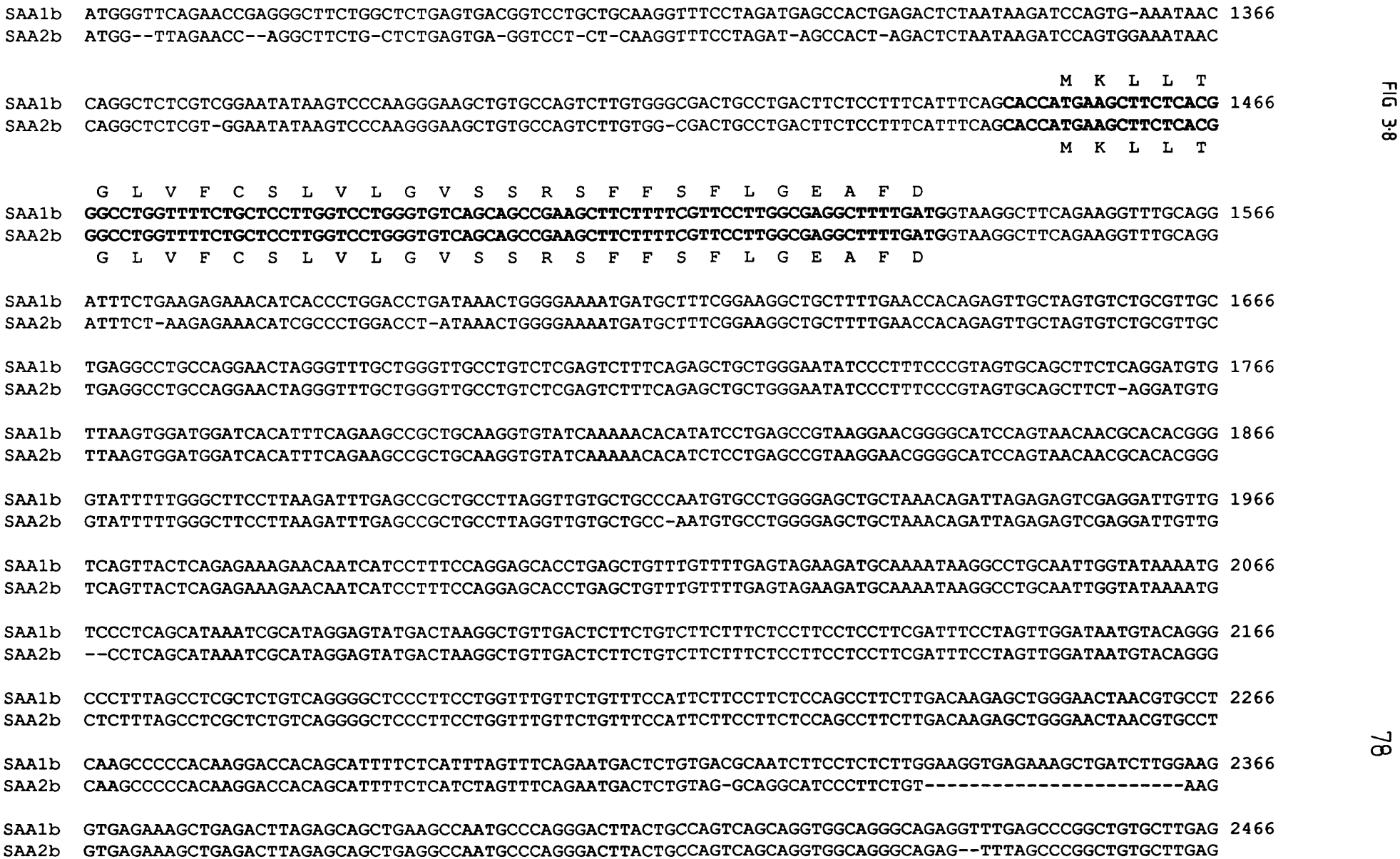
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M K L L T
SAA1b CAGGCTCTCGTCGGAATATAAGTCCCAAGGGAAGCTGTGCCAGTCTTGTGGGCGACTGCCTGACTTCTCCTTTCATTTCAGCACCATGAAGCTTCTCACG 1466
SAA2b CAGGCTCTCGT-GGAATATAAGTCCCAAGGGAAGCTGTGCCAGTCTTGTGG-CGACTGCCTGACTTCTCCTTTCATTTCAGCACCATGAAGCTTCTCACG
M K L L T
G L V F C S L V L G V S S R S F F S F L G E A F D
SAA1b GGCCTGGTTTTCTGCTCCTTGGTCCTGGGTGTCAGCAGCCGAAGCTTCTTTTCGTTCCTTGGCGAGGCTTTTGATGGTAAGGCTTCAGAAGGTTTGCAGG 1566
SAA2b GGCCTGGTTTTCTGCTCCTTGGTCCTGGGTGTCAGCAGCCGAAGCTTCTTTTCGTTCCTTGGCGAGGCTTTTGATGGTAAGGCTTCAGAAGGTTTGCAGG
G L V F C S L V L G V S S R S F F S F L G E A F D
SAA1b ATTTCTGAAGAGAAACATCACCCTGGACCTGATAAACTGGGGAAAATGATGCTTTCGGAAGGCTGCTTTTGAACCACAGAGTTGCTAGTGTCTGCGTTGC 1666
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SAA1b TGAGGCCTGCCAGGAACTAGGGTTTGCTGGGTTGCCTGTCTCGAGTCTTTCAGAGCTGCTGGGAATATCCCTTTCCCGTAGTGCAGCTTCTCAGGATGTG 1766
SAA2b TGAGGCCTGCCAGGAACTAGGGTTTGCTGGGTTGCCTGTCTCGAGTCTTTCAGAGCTGCTGGGAATATCCCTTTCCCGTAGTGCAGCTTCT-AGGATGTG
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SAA2b --CCTCAGCATAAATCGCATAGGAGTATGACTAAGGCTGTTGACTCTTCTGTCTTCTTTCTCCTTCCTCCTTCGATTTCCTAGTTGGATAATGTACAGGG
SAA1b CCCTTTAGCCTCGCTCTGTCAGGGGCTCCCTTCCTGGTTTGTTCTGTTTCCATTCTTCCTTCTCCAGCCTTCTTGACAAGAGCTGGGAACTAACGTGCCT 2266
SAA2b CTCTTTAGCCTCGCTCTGTCAGGGGCTCCCTTCCTGGTTTGTTCTGTTTCCATTCTTCCTTCTCCAGCCTTCTTGACAAGAGCTGGGAACTAACGTGCCT
SAA1b CAAGCCCCCACAAGGACCACAGCATTTTCTCATTTAGTTTCAGAATGACTCTGTGACGCAATCTTCCTCTCTTGGAAGGTGAGAAAGCTGATCTTGGAAG 2366
SAA2b CAAGCCCCCACAAGGACCACAGCATTTTCTCATCTAGTTTCAGAATGACTCTGTAG-GCAGGCATCCCTTCTGT----------AAG
SAA1b GTGAGAAAGCTGAGACTTAGAGCAGCTGAAGCCAATGCCCAGGGACTTACTGCCAGTCAGCAGGTGGCAGGGCAGAGGTTTGAGCCCGGCTGTGCTTGAG 2466
SAA2b GTGAGAAAGCTGAGACTTAGAGCAGCTGAGGCCAATGCCCAGGGACTTACTGCCAGTCAGCAGGTGGCAGGGCAGAG--TTTAGCCCGGCTGTGCTTGAG

FIG 3-8


SAA1b GTCAGGGCTCTTGCCAGGTAGACGCATCACTGACCACCTCCTAGAGGTTGATGGTTATGAATCTCAGGCACACCTTGGCATCACCTGAAATACCCATGCC 2566
SAA2b GTCAGGGCTCTTGCCAGGTAGACGC-TCACTGACCACCTCCCAGAG-TTGATGGTTAT-AATCTCAGACACACCTTGGCATCACCT-AACTACCCATGCC
SAA1b TTCAACTCCCCAGCAGAGTCTGCAGAAACTGGCCTGGGGGTGTGGCCTGGGCACTGGGTACTTTCAGTTTC------TCTCTGGGTGATTAGAAAGTGCAG 2660
SAA2b TTCAACTCCCCAGCAGAGTCTGCAGAAACTGGCCTGGGGGTGTGGCCTGGGCACTCGGGACTTTCAGTTTCAGTTTCTCTCTGGGTGATTAGAAAGTGCAG
SAA1b CCAAGGCTCACGCCTGTAATTCCAGCACTTTGGGAGGCCAAGGTGGATGAATCACTTGAGGTCATGAGTTCCGGAGCAGCCTGGCCAACATGGTGAAACC 2760
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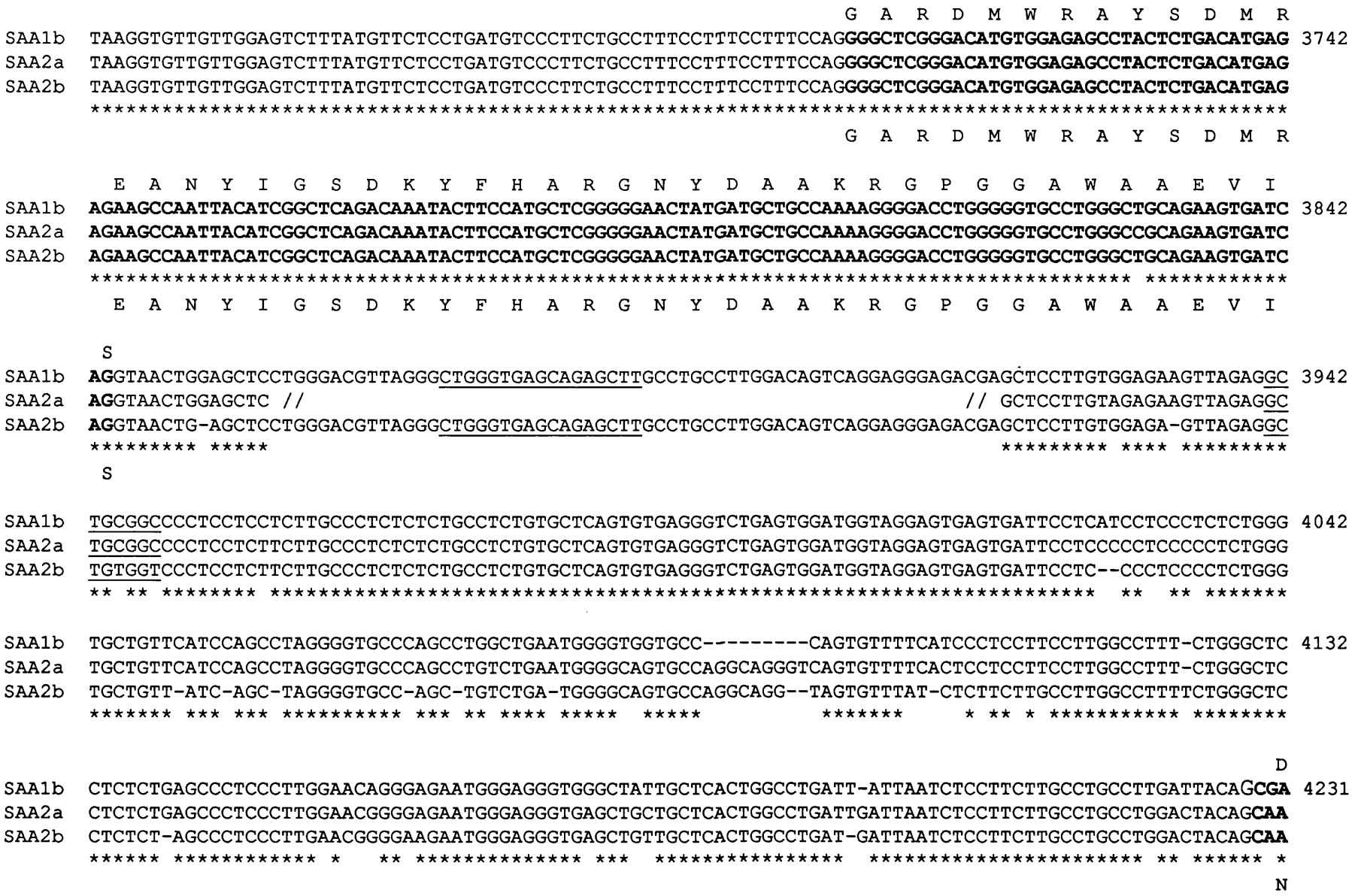
SAA1b CCGTCTCTACTAAAAATACTAAAATGTAGCCAGGCGTGGTGGCAGGCACCTGTAATCCCAGCTACTCAGGAGGCTGAAGCACGAGAATCACTTCAACCCG 2860
SAA2b CTGTGTCTACTAAAAATATGAAAATGTAGCCAGGCGTGGTGGCAGGCAC-TGTAATCCCAGCTACTCAGGAGGCTGAAGCAGGAAAAT-ACTTGAACC-G
SAA1b AGAAGCAGAGGTTGCAGTGACTAGAGATCGCACCAGTGTCCTCCAACCTGGGTGACAGA---GCGAGACT--CCATCTAAAAAAAATGAAAAAGAAAGTG 2955
SAA2b GGAAGCGGAAGTTGTAGTGAGTT-ACATTGCTCCAGTGTGCTCCA-CCTGG-TGACAGAAGTGCAAGACATGCCGTCTAAAAAAATAAAAAAAGAAAAAA
SAA1b -----CAGCCAAGGCAGAGCACCACTG--CCCTATTGCTTCCTCAAGCAACCCACAGCATCAGTACAGCCTACTAAGAAAGTATTTAGGGACTTTTATGC 3048
SAA2b AAAGTCAACCACGGCGGAGAACCGCTAGACTCTATTGCTTCCTCAAGCAACCCACAGTATCAGTACAGCCTACTGAGAAAGTGTTTAGGGACTTTTATGC
SAA1b TCCTAACAGTCACTGGAACTCACGTCACAATGACGTGTATTCCATTTGCAAGAATATATACTTTAGGTCGGGGTGCGGTGGCTCACGCCTGTAATCCCAG 3148
SAA2b TCCTAACAGTCACTGGAACTCAGGTCACAATGACATGTATTC-ATTTGCAAGA-TATATACTTTAGCT----------
SAA1b CACTTTGGGAGGCCAAGGCAGGGGGATCACGAGGTCAGGAGTTCGAGACCAGCCTGACCAACATGGTGAAATCCCCGTCTCTACTAAAAATACAAAAATT 3248
SAA2b ----------
SAA1b AGCCAGGCGTGATGGCGCATGCCTGTAATCTCAGCTACTCAGGAGGCTGATCGGAAGAAGAATCTCTTGAACCTGGAGGTGGAGGTTGCGATGAGCTGAG 3348
SAA2b ----------
SAA1b ATAGCACCACTGCAACTCCAGCCTGGGCGACAGAACTTTAGTAGTCAGGGCAGAAG-TACTC-T-GT-GTC-TGCCACCTTTCTCAGCATCAGTATTCCA 3443
SAA2b ----------AGTCAGGGCAGAAGCTACTCCTGGTCGTCCTGCCACCTTTCTCAGCATCAGTATTCCA
SAA1b TGTCACTACCTCATTCATACACACTCCTGGATCTTATCATAGGCAGCTTCATTCTATAGCAGTGGCTCTTCACCAGGGCACTTGAAGAAGCCAACTAGGA 3543
SAA2b TGTCACCACCTCATTCATATACACTCCTGGATCTTATCATAGGCAGCTTCATTCTATAGCAGTGGCTCTTCACCAGGGCACTTGAAGAAGCCAACTAGGA
SAA1b TAAAGGAATGTGCTTCTCAACCCATGGTATCCAAGGCTGCTATGATCACAGGCTGAAAGCTTGAAGTCAGTGGAAGATTTGTCCTTCCT-CATTCCCCTC 3642
SAA2a ATGCTTGAAGTCAGTGGAAGATTTGTCCTTCCT-CATTCCCCTC
SAA2b TAAAGGAATGTGCTTCT-AACCCATG-TATCCA-GGCTGCTATGATCACAGGCTGAAAGCTTGAAGTCAGTGGAAGATTTGTCCTTCTTTCATTCCCCTC
* ********** * **********

FIG 3·8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SAA1b TAAGGTGTTGTTGGAGTCTTTATGTTCTCCTGATGTCCCTTCTGCCTTTCCTTTCCTTTCCAGGGGCTCGGGACATGTGGAGAGCCTACTCTGACATGAG 3742
SAA2a TAAGGTGTTGTTGGAGTCTTTATGTTCTCCTGATGTCCCTTCTGCCTTTCCTTTCCTTTCCAGGGGCTCGGGACATGTGGAGAGCCTACTCTGACATGAG
SAA2b TAAGGTGTTGTTGGAGTCTTTATGTTCTCCTGATGTCCCTTCTGCCTTTCCTTTCCTTTCCAGGGGCTCGGGACATGTGGAGAGCCTACTCTGACATGAG
G A R D M W R A Y S D M R
E A N Y I G S D K Y F H A R G N Y D A A K R G P G G A W A A E V I
SAA1b AGAAGCCAATTACATCGGCTCAGACAAATACTTCCATGCTCGGGGGAACTATGATGCTGCCAAAAGGGGACCTGGGGGTGCCTGGGCTGCAGAAGTGATC 3842
SAA2a AGAAGCCAATTACATCGGCTCAGACAAATACTTCCATGCTCGGGGGAACTATGATGCTGCCAAAAGGGGACCTGGGGGTGCCTGGGCCGCAGAAGTGATC
SAA2b AGAAGCCAATTACATCGGCTCAGACAAATACTTCCATGCTCGGGGGAACTATGATGCTGCCAAAAGGGGACCTGGGGGTGCCTGGGCTGCAGAAGTGATC
E A N Y I G S D K Y F H A R G N Y D A A K R G P G G A W A A E V I
S
SAA1b AGGTAACTGGAGCTCCTGGGACGTTAGGGCTGGGTGAGCAGAGCTTGCCTGCCTTGGACAGTCAGGAGGGAGACGAGCTCCTTGTGGAGAAGTTAGAGGC 3942
SAA2a AGGTAACTGGAGCTC // // GCTCCTTGTAGAGAAGTTAGAGGC
SAA2b AGGTAACTG-AGCTCCTGGGACGTTAGGGCTGGGTGAGCAGAGCTTGCCTGCCTTGGACAGTCAGGAGGGAGACGAGCTCCTTGTGGAGA-GTTAGAGGC
S
SAA1b TGCGGCCCCTCCTCCTCTTGCCCTCTCTCTGCCTCTGTGCTCAGTGTGAGGGTCTGAGTGGATGGTAGGAGTGAGTGATTCCTCATCCTCCCTCTCTGGG 4042
SAA2a TGCGGCCCCTCCTCTTCTTGCCCTCTCTCTGCCTCTGTGCTCAGTGTGAGGGTCTGAGTGGATGGTAGGAGTGAGTGATTCCTCCCCCTCCCCCTCTGGG
SAA2b TGTGGTCCCTCCTCTTCTTGCCCTCTCTCTGCCTCTGTGCTCAGTGTGAGGGTCTGAGTGGATGGTAGGAGTGAGTGATTCCTC--CCCTCCCCTCTGGG
SAA1b TGCTGTTCATCCAGCCTAGGGGTGCCCAGCCTGGCTGAATGGGGTGGTGCC---------CAGTGTTTTCATCCCTCCTTCCTTGGCCTTT-CTGGGCTC 4132
SAA2a TGCTGTTCATCCAGCCTAGGGGTGCCCAGCCTGTCTGAATGGGGCAGTGCCAGGCAGGGTCAGTGTTTTCACTCCTCCTTCCTTGGCCTTT-CTGGGCTC
SAA2b TGCTGTT-ATC-AGC-TAGGGGTGCC-AGC-TGTCTGA-TGGGGCAGTGCCAGGCAGG--TAGTGTTTAT-CTCTTCTTGCCTTGGCCTTTTCTGGGCTC
D
SAA1b CTCTCTGAGCCCTCCCTTGGAACAGGGAGAATGGGAGGGTGGGCTATTGCTCACTGGCCTGATT-ATTAATCTCCTTCTTGCCTGCCTTGATTACAGCGA 4231
SAA2a CTCTCTGAGCCCTCCCTTGGAACGGGGAGAATGGGAGGGTGAGCTGCTGCTCACTGGCCTGATTGATTAATCTCCTTCTTGCCTGCCTGGACTACAGCAA
SAA2b CTCTCT-AGCCCTCCCTTGAACGGGGAAGAATGGGAGGGTGAGCTGTTGCTCACTGGCCTGAT-GATTAATCTCCTTCTTGCCTGCCTGGACTACAGCAA
N

FIG 3-8

Figure 3.8 **Nucleotide Sequences of Human SAA Genes.**
The complete nucleotide sequence for *SAA1ß* is shown aligned with that for *SAA2ß* (Woo *et al*, 1987; Edbrooke *et al*, 1989) and the sequence obtained for *SAA2α*, identity between all 3 sequences is indicated by an asterisk. Sequence numbering for *SAA1ß* starts at the XbaI site 825bp upstream of exon 1. Exons are shown in bold type with derived amino acid sequences for SAA1 and SAA2 shown above and below the nucleotide sequence respectively; the His/Arg SAA2 polymorphism occurs at residue 71 in exon 4. The underlined sequences 5' of exon 1 represent the NF-IL6, CTGGGA, NFκB and TATA box homologies, the putative poly(A) signal in the 3'UT is also underlined. Sequences underlined in intron 3 represent homologies to *chi*-like sequences (see discussion). Direct repeats flanking *Alus* are double underlined. The 5' limit of homology between *SAA1* and *SAA2* occurs at nucleotide 380. The 3' limit of homology was extended with the addition of a 25bp deletion into the 3'UT of *SAA1*.

CpG dinucleotide (seen as a G to A change if the mutation occurred on the complementary strand) which are recognized as hot spots of mutation in the genome due to deamination of 5-methylcytosine (Bird, 1980). Although there were several differences within the gene between *SAA2α* and *SAA2ß* their allelic status was predicted by the strong similarity in the 3' flanking region and analysis of the above mentioned RFLPs in family and population studies (section 3.2.1/2).

Over 1000nt of *SAA2α* intron 2 and 3 sequence was obtained revealing 7% sequence variation compared to the same regions in *SAA2ß*. This compares to 99% conservation in exons 3 and 4 extending to the putative poly(A) signal and at least 24nt beyond. The higher level of intronic variation is not unexpected due to the relaxed selective pressure in these regions. When *SAA1ß* is compared to *SAA2* 91.2% sequence conservation is evident over all 4 exons extending to beyond the poly(A) signal. This homology increases to 97.6% if the 5' and 3' untranslated regions are omitted from the calculation. The intronic nucleotide variation is comparable to that seen between *SAA2α* and *SAA2ß*. This strong homology among non-allelic genes implies that a mechanism such as gene conversion may have occurred at some stage during their evolution (see sections 1.4 and 4.2 for further discussion).

3.2.4 Isolation of Unique Sequences for Chromosome Walking.

In order to characterize sequences immediately flanking those already cloned the technique of chromosome walking can be employed. By using unique sequences derived from the ends of genomic clones to rescreen a library, clones containing this sequence but also extending beyond those delineated will be obtained.

In order to identify reiterated sequences within λ1.1, 1.2, 1.3, 2.1 and 2.2 such as *Alu* repeats and L1 repeats Southern clone blots were hybridized with ^{32}P-labelled total human genomic DNA restricted with HindIII. Repetitive regions identified by this method are shown in figure 3.6. The regions underlined in figure 3.6 were subcloned for use as probes, namely a 4.0kb SstI fragment from λ1.1, a 5kb EcoRI/BamHI fragment from λ1.3, a 1.0kb BamHI/SstI fragment from λ2.1 and a 2.5kb XbaI/SalI fragment from λ2.2. The subclones were further mapped and fragments (λ1L, λ1R, λ2L and λ2R) containing non-repetitive DNA isolated as shown in figure 3.9.

Each of the left and right probes isolated were subsequently used as probes against HindIII genomic Southern blots to confirm that they were unique. The probes hybridized to single HindIII fragments as follows:

i) λ1L hybridized to a unique 2.7kb band confirming its position on the genomic clone map as the NcoI fragment within a 2.7kb HindIII fragment, although a HindIII site occurs within the λ1L fragment a second band was not easily identifiable.

ii) λ1R hybridized to a 4.0kb HindIII fragment, again confirming the mapping data. In addition this probe was hybridized to other genomic digests detecting a 10kb NcoI, confirming the restriction map, and a 5kb BglII fragment which extends beyond λ1.3.

iii) λ2L hybridized to a 9kb HindIII fragment, slightly smaller than the fragment

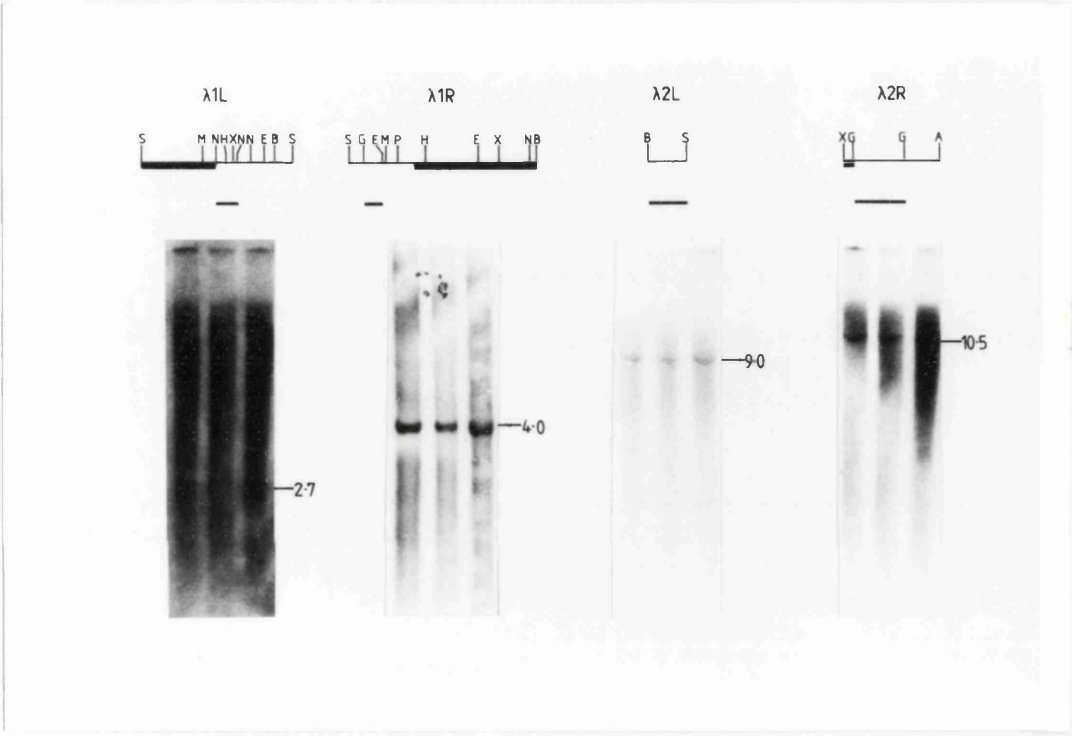


Figure 3.9 Characterization of Unique Intergenic Sequences.
Restriction maps are shown for fragments isolated from the left and right ends of the λ clones indicated at the top. The solid regions represent fragments which hybridized to total human genomic DNA probe and contain repetitive sequences. The bars below each map show the fragments labelled to probe HindIII Southern blots. Examples of the resulting autoradiographs with the hybridizing fragments in kb are shown.

hybridizing to pA1. λ2L is within a HindIII fragment extending to the end of λ2.1, indicating that the clone terminates within a 9kb HindIII fragment.
iv) λ2R hybridized to a 10.5kb HindIII band, indistinguishable in size to that hybridizing to pA1 and which extends beyond the end of λ2.2 (figure 3.9).

These results were important in demonstrating that firstly, the probes isolated were unique sequences and secondly, that the λ clone inserts were colinear with genomic sequences and not rearranged (at least in the regions from which the probes were derived). However despite the steps taken to isolate unique sequences, each of the probes still gave some background hybridization presumably due to low levels of repeats still present or the inability to separate the fragments completely from contaminating repetitive sequences present elsewhere in the subclones. The λ2R fragment was recloned to reduce the likelihood of the latter effect occurring.

3.2.5 Screening of Cosmid Genomic Libraries: Isolation of *SAA4*.

Comparison of restriction fragments from the SAA genomic clones with the hybridizing bands on genomic Southerns indicated that there were further SAA genes which had not been cloned. In addition an understanding of the mechanisms involved in SAA gene family evolution would be enhanced by isolating intergenic sequences to determine the relative orientation of the genes. One approach to achieve this was the screening of cosmid libraries which contain genomic DNA inserts in the region of 40kb in length using both SAA specific probes and probes containing unique sequences from the ends of the λ clones to "walk" along the chromosome. In this way the likelihood of obtaining contiguous clones would be greatly increased over screening λ libraries.

A human acute lymphocytic leukaemia cell line (HPB-ALL) Sau3A-partial library in the vector cos202 containing 35-40kb genomic DNA inserts (Kioussis *at al*, 1987) was screened using a combination of chromosome walking and SAA cDNA probes. The cos202 library was supplied as 4 individually amplified aliquots. $2x10^5$ clones were plated on each of 2 Hybond-N 20x20cm filters as described in "Materials and Methods". Duplicate filters were hybridized with ^{32}P-labelled pA1 and hybridized at 65°C overnight. Membranes were washed to 1xSSC, 0.1% SDS at 65°C and autoradiographed overnight. Eight duplicate hybridizing clones were identified and picked, two of these came through secondary and tertiary screening.

Cosmid DNA was prepared from these recombinants and subjected to restriction digestion, southern blotting and hybridization to pA1. The enzymes HindIII, BamHI, EcoRI, SstI, NcoI, BglII, PstI, XbaI, XhoI and KpnI gave a hybridization pattern to pA1a and pA1b which was indistinguishable from *SAA2ß*. However further bands were also evident which hybridized less strongly to both probes but corresponded to hybridizing fragments in genomic DNA digests. pA1a, pA1b, λ2R (a walking probe derived from λ2.2, see above) and fragments from the ampicillin-resistance and hygromycin-resistance genes present in the cos202 vector were used as probes to construct a physical map for cos2.3 (fig. 3.10 and fig. 3.11). The insert was 35kb long with a unique SAA-hybridizing

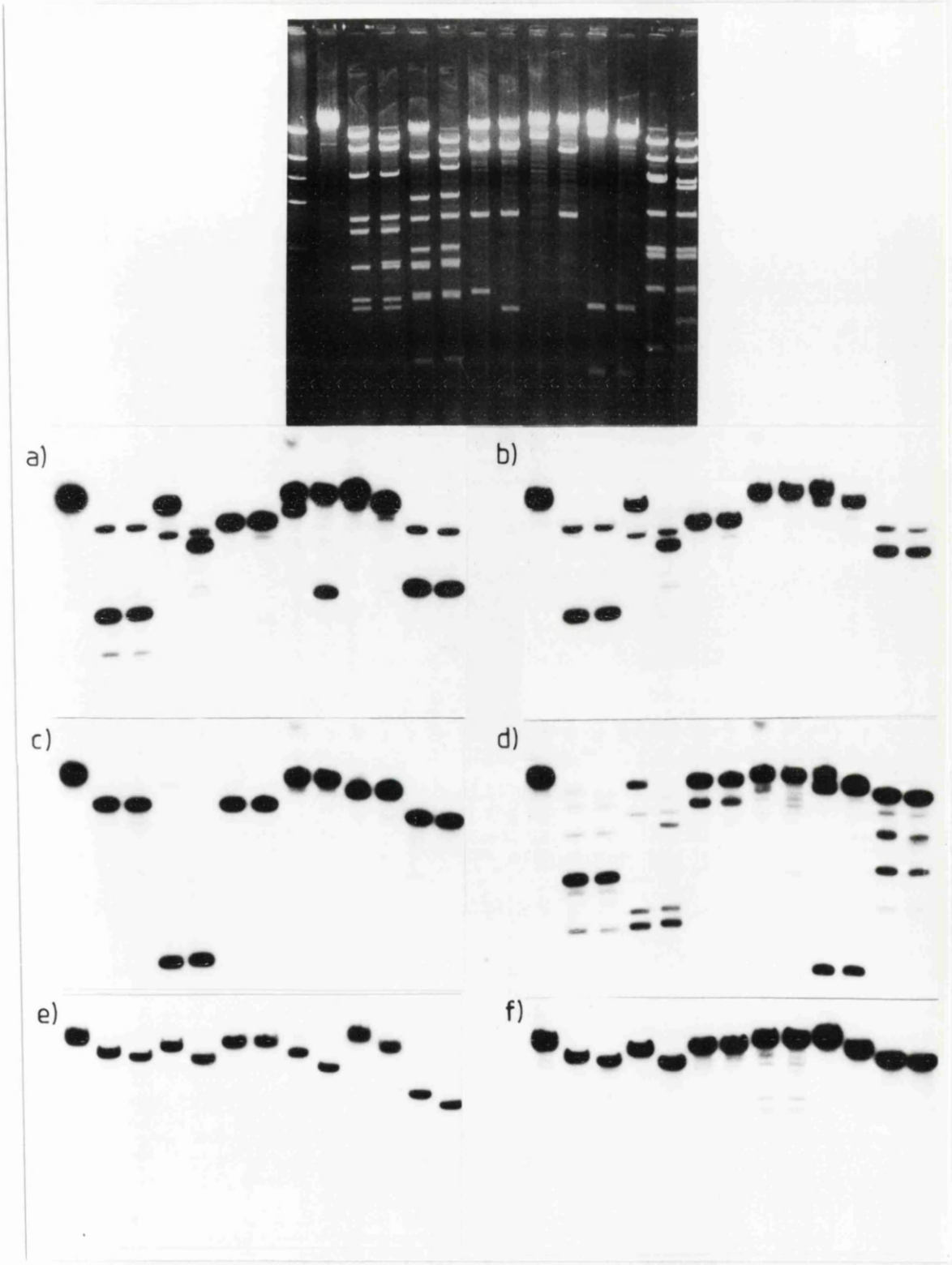


Figure 3.10 Restriction Mapping of Cos2.3.
Examples of digests of Cos2.3 DNA (from left to right) BamHI, HindIII, BamHI+HindIII, BglII, BamHI+BglII, KpnI, BamHI+KpnI, XhoI, BamHI+XhoI, XbaI, BamHI+XbaI, SstI, BamHI+SstI. Fragments were electrophoresed with HindIII digested λDNA markers (first lane). The gel was blotted and probed with a) pA1a, b) pA1b, c) lambda2R, d) cos2.3 3.5kb HindIII fragment, e) AvaII Amp^R gene fragment, f) EcoRI Hyg^R gene fragment.

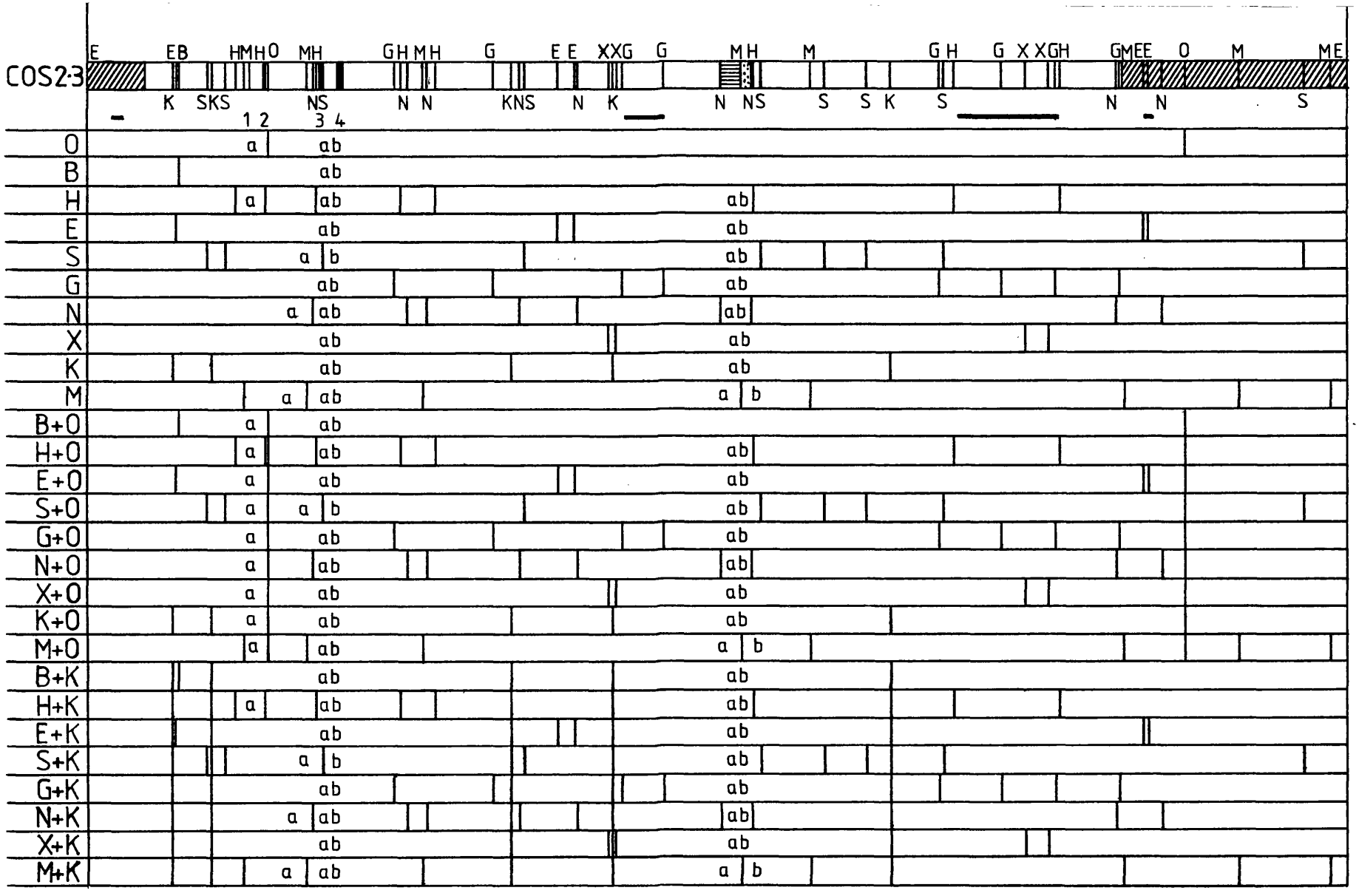


Figure 3.11 Construction of cos2.3 Restriction Map.

Examples of restriction digests are shown with hybridization to pA1a (a) and pA1b (b). The other probes used in mapping shown as bars under the map were (from left to right) Amp^R AvaII fragment, λ2R, HindIII 3.5kb fragment and Hyg^R EcoRI fragment. *SAA2* exons are numbered and shown as solid boxes. The minimal fragments hybridizing to pA1a (horizontal hatch) and pA1b (dotted) for the *SAA4* locus are shown. Diagonal hatching represents the cosmid vector.

region mapping to 10kb downstream of *SAA2ß* which was designated *SAA4*. The region overlapping with λ2.1 and 2.2 was identical on the basis of restriction mapping (with the exception of the polymorphisms previously described). The HindIII fragment containing λ2R (section 3.2.4) also contained *SAA4*, a result predicted by Southern analysis. In addition a further 15-20kb of 3' sequence flanking *SAA4* was present in this clone extending the characterized region for this part of the SAA locus to 40kb. The λ1L and λ1R walk probes did not hybridize to cos2.3 (data not shown) thus it was not possible to establish contiguity between the *SAA1* and *SAA2/SAA4* loci.

In addition the cos202 library filters were screened with a "cocktail" of the 4 walking probes, however this was unsuccessful producing hybridization to a large proportion of clones probably due to the low level of repeats present.

3.2.6 Calculation of SAA Gene Dosage.

In order to calculate the complexity of the human SAA gene family restriction fragments from cloned DNAs were assigned to those on genomic Southern blots. The complete maps of the cloned genes are shown in figure 3.12, assignment of each of these loci to Southerns is shown in figure 3.13 for each of the restriction digests carried out. The structure of the clone described by Sack and colleagues was also included using data from the published map of clone GSAA1 (Sack and Talbot, 1989) which was designated *SAA3*.

Taking HindIII digests as an example, genomic DNA produces the pA1-hybridzing bands previously described (figure 3.1). The 10.5kb fragment represents hybridization to *SAA4*, the 3.6kb band is present in *SAA3* and the 1.3kb fragment corresponds to *SAA1*. The 4.6 and 3.0kb fragments are represented by the *SAA2α* and *SAA2ß* clones respectively, the polymorphic HindIII site being present at the 3' end of intron 2 as described above. In a similar manner assignments could be made for the other restriction digests. It was also evident that the observed BglI RFLPs were due to polymorphisms within the *SAA1* gene although variants were not obtained in the screening carried out as part of these studies. In each digest all bands can be accounted for by the cloned sequences, thus excluding the possibility that highly homologous genes are present within one band in all the digests, the human SAA gene family comprises four members. Three of these have been cloned in this study: *SAA1ß*, *SAA2α* and *SAA4*, *SAA3* and an *SAA2* allele (ß) have been described elsewhere (Sack and Talbot, 1989; Woo *et al*, 1987).

3.2.7 SAA Genes: Intra- and Inter-Species Sequence Comparisons.

3.2.7.1 Nucleotide Sequence Comparisons.

The human SAA genes comprise four discrete loci, two are highly homologous (*SAA1* and *SAA2*) while *SAA3* and *SAA4* are diverged from these. This was evident from both nucleotide sequence data for *SAA1-3* and hybridization intensity to the *SAA1* probe. The homology between the exons of *SAA1* and *SAA2* was >97% for exons 1-3 and 94% in exon 4. *SAA3* shows maximal homology of 89% in exon 3 to *SAA1/2* (table 3.3). The

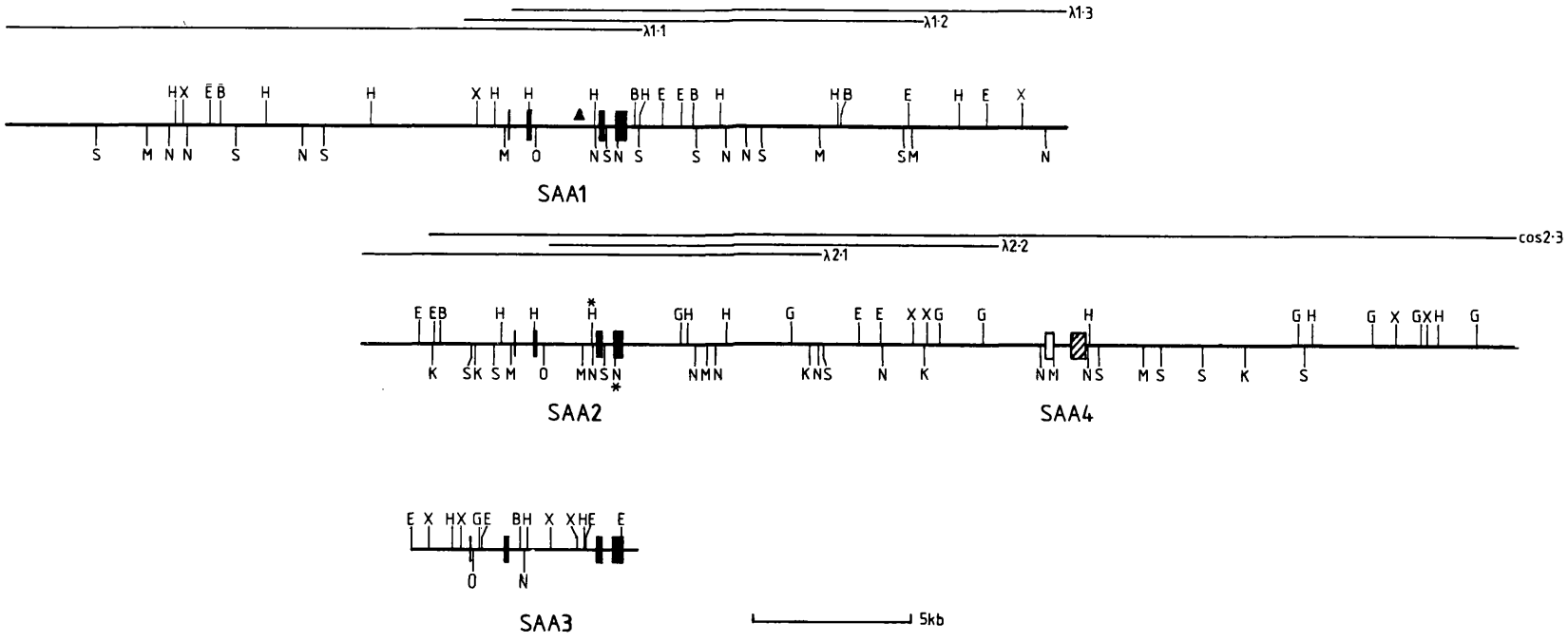


Figure 3.12 Alignment of Human SAA Genomic Clones.
The complete maps are shown for the SAA genomic clones obtained to date with *SAA1-3* aligned at exon 3. *SAA4* regions hybridizing to pA1a (open box) and pA1b (hatched box) were delimited through mapping of a subclone. * denotes polymorphic site, filled triangle = position of *SAA1AluB* insertion. Abbreviations for restriction sites as figure 3.4. The *SAA3* map was compiled using data from Sack and Talbot (1989).

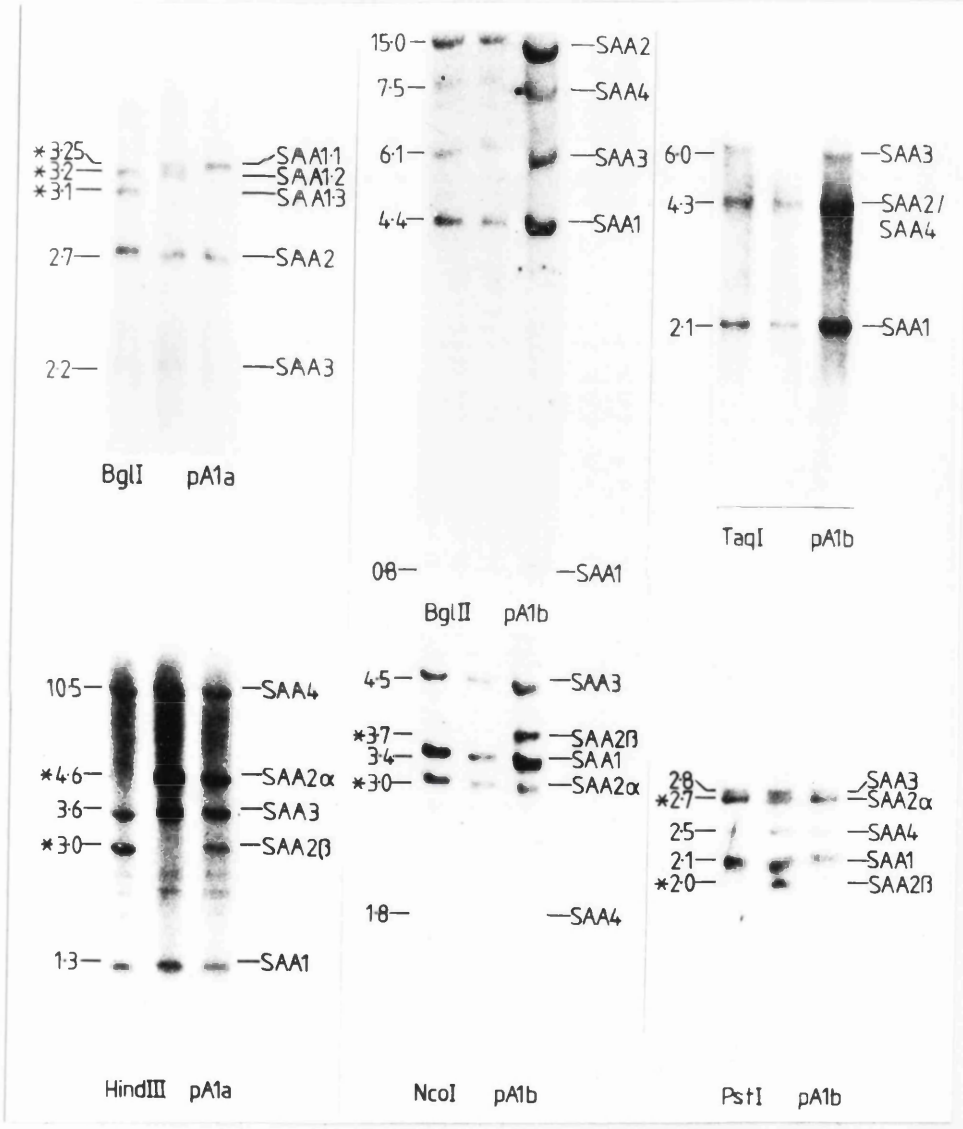


Figure 3.13 Assignment of SAA Loci to Hybridizing Fragments on Southerns. Selected autoradiographs of Southern blots with the restriction digest and probe used are shown. Fragments were assigned to cloned SAA loci on the basis of restriction mapping and cloning data as shown to the right. *SAA3* was assigned using the data from publications by Sack (1983); Sack *et al* (1988) and Sack and Talbot (1989).

homology between *SAA1* and *SAA2* introns 1, 2 and 3 was 93, 74 and 90% respectively, with the homology for intron 2 rising to 93% if *SAA1*AluB was removed from the calculation. In comparison to *SAA3*, *SAA1/SAA2* were 40% homologous in the 5'-flanking region to -450bp (*SAA1* and *SAA2* were 88% homologous in this region) and in intron 3. No significant similarity occurred in introns 1 and 2 except that *SAA3* also contains an *Alu* repeat (see below).

Table 3.3 Nucleotide Sequence Homologies Between Human SAA Genes.

	5'	Exon 1	2	3	4	Intron 1	2	3	3'
SAA2	88	97	100	99	94	93	74	90	70
SAA3	40	-	79	89	85	-	-	40	63

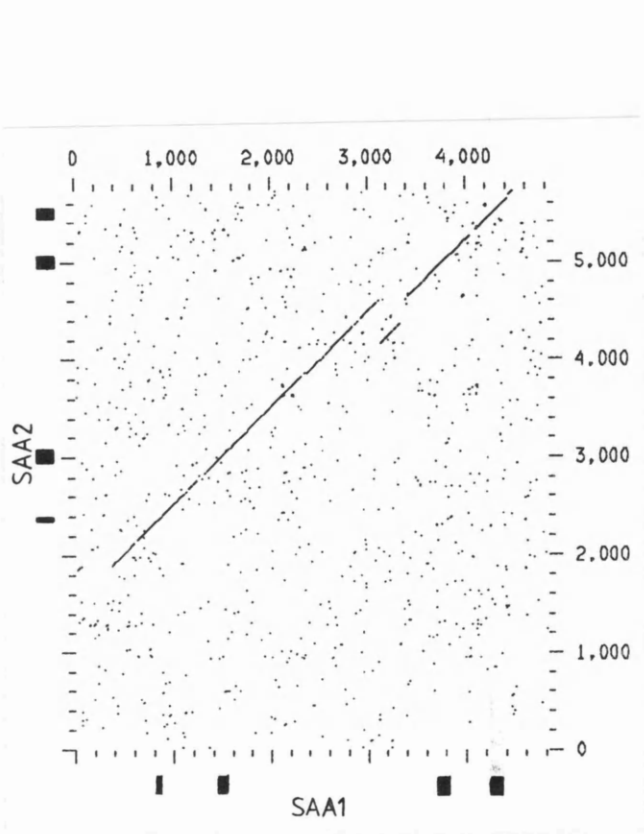
The maximum nucleotide homologies were calculated between SAA1ß and SAA2ß or SAA3, these are shown as the percentage of identical nucleotides over the regions indicated. 5' = region extending 450bp 5' of exon 1; 3' = 3'UT to SAA1 poly(A) signal; - = negligible homology.

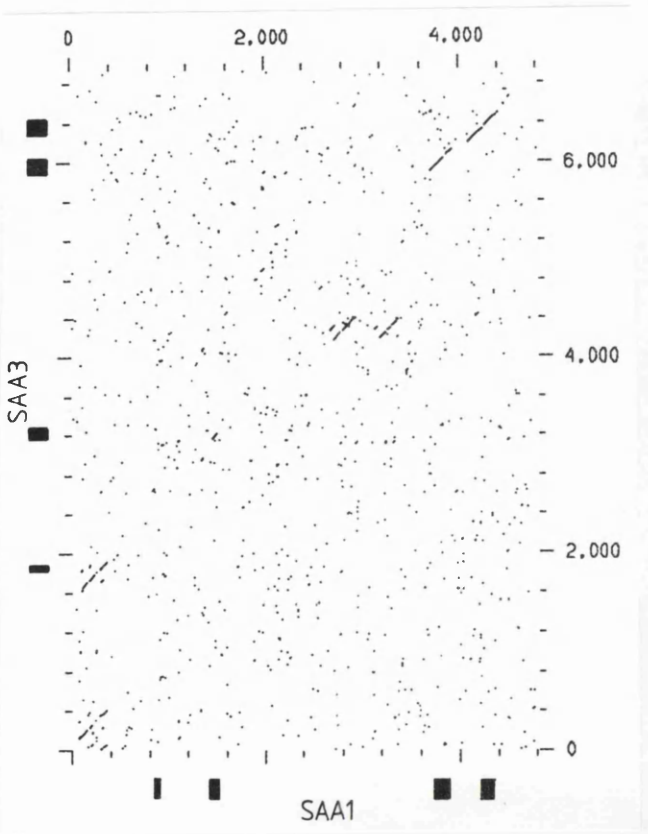
The SAA genes in mice are structurally similar to those in humans with strong homology of *SAA1* and *SAA2* but not *SAA3/SAA4* (Lowell *et al*, 1986a; Yamamoto *et al*, 1987). Comparison of the human and murine sequences using GCG bestfit revealed no homology with the exception of the exons and small regions surrounding them (for *hSAA1/SAA2* versus *mSAA1/SAA2*). The complete *mSAA3* sequence was not available on the database to compare the *SAA3* genes between the two species, however comparison of *hSAA3* with *mSAA1/2* reveals slightly less homology than between the human and murine *SAA1/2* homologues (see fig 3.14 for examples of dotplots obtained).

Human *SAA1-3* genes have *Alu* repeats in intron 2, each being flanked by direct repeats, believed to be duplications of the insertion target sequence (see section 5.2). An alignment of the *Alu* repeats with a recent consensus of 125 *Alu* repeats is shown in figure 3.15. By recording nucleotide substitutions at diagnostic positions it was possible to assign the SAA *Alu* repeats to the "Sa" subclass (Jurka and Smith, 1988). This indicates that the repeats in the second introns of the human SAA genes were probably inserted during the same evolutionary period.

3.2.7.2 Phylogenetic Comparison of SAA/AA Proteins.

Comparison of proteins from a number of species at the primary sequence level can reveal phylogenetic conservation of domains with potential functional significance. The amino acid and derived amino acid sequences for the 17 known SAA or AA proteins


0
1,000
2,000
3,000
4,000
5,000
4,000
3,000
2,000
1,000
0
SAA2
SAA1


0
2,000
4,000
6,000
4,000
2,000
0
SAA3
SAA1

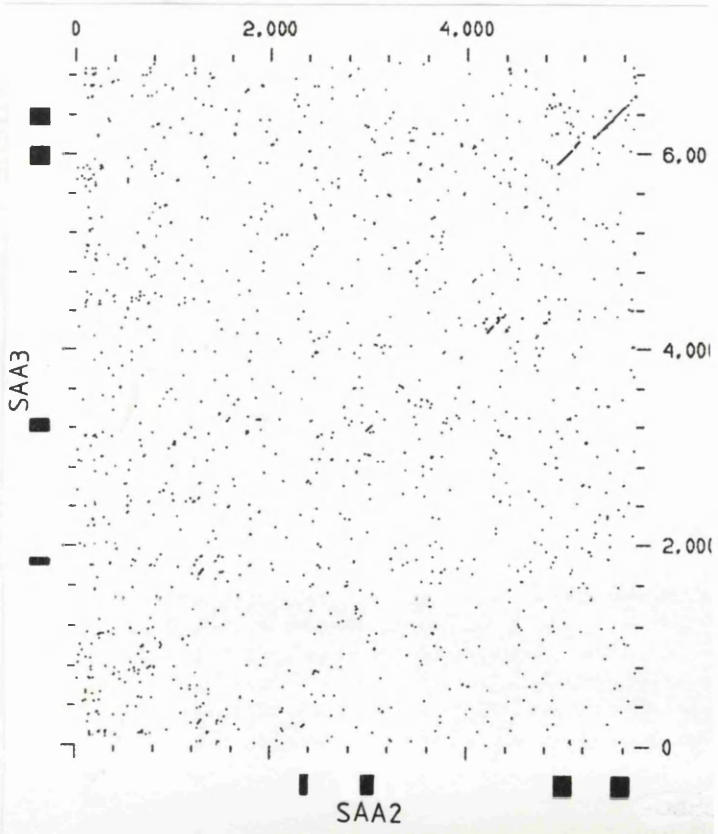

0
2,000
4,000
6,00
4,00
2,00
0
SAA3
SAA2

FIGURE 3·14A

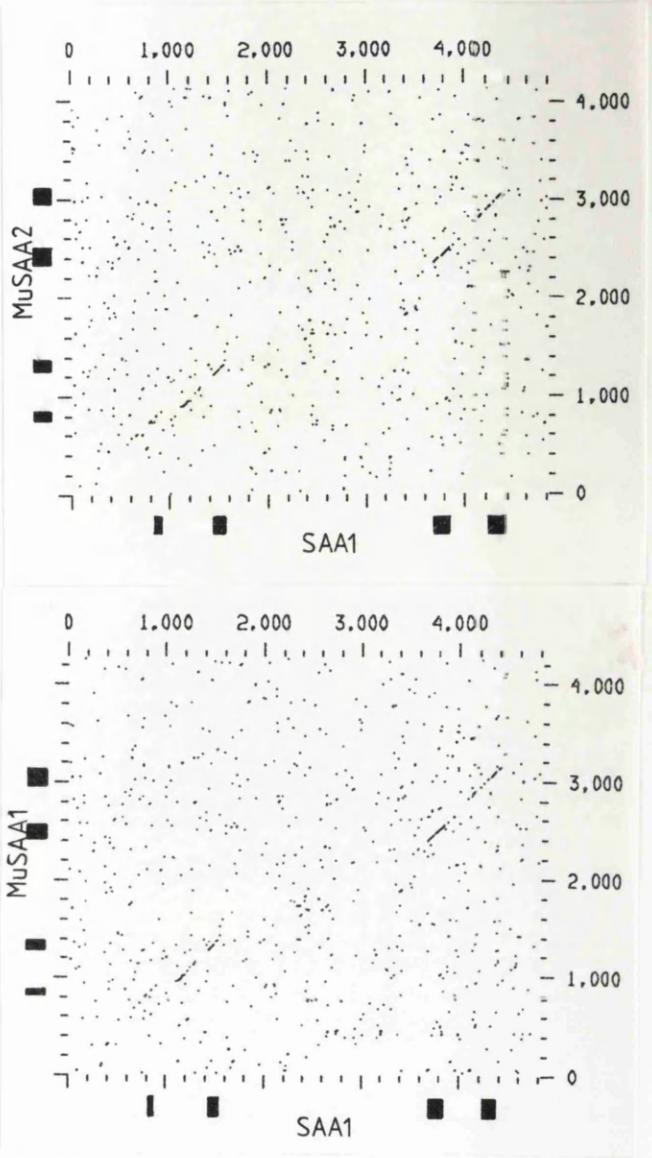


Figure 3.14 Dotplot of Human and Murine SAA Gene Sequences.
Genomic sequences for SAA genes were analyzed using the COMPARE and DOTPLOT programmes of the GCG package.
A) Human genes. (Previous page; from left to right) *SAA1* v. *SAA2*, *SAA1* v. *SAA3*, and *SAA2* v. *SAA3*. Exons are shown as boxes with the nucleotide numbering for *SAA1* as figure 3.9. *Alu* sequences are visible between nucleotides 2400-3400 of *SAA1* as well as 0-400 (seen in comparison with *SAA3*).
B) Murine genes. Comparison of human *SAA1* with murine *SAA1* (lower) and *SAA2* (upper). Homology is limited to intron 1 and exons 2, 3 and 4.

```
SAA1AluA         AGAAAGTGCAGCCAAGGCTCACGCCTGTAATTCCAGCACTTTGGGAGGCCAAGGT
SAA2Alu          AGAAAGTGCAGCCAAGGCTCACGCCTGTAATTCCAGCACTTTGGGTGGCCAAGGT
SAA1AluB    ACTTTAGGTCGGGGTGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCAAGGC
SAA3Alu     G----AGA-------------------------AAT----ATACCCCGAG-GGC-----T
ConsAlu           GGCCGGG-CGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGC
                  *                            ***      **   * * ***

SAA1AluA    GGATGAATCACTTGAGGTCATGAGTTCCGGAGCAGCCTGGCCAACATGGTGAAA-CCCCG
SAA2Alu     GGATGAATCACTTGAGGTCATGAGTTCCGGAGCAGCCTGGCCAACATGGTGAAA-CCCTG
SAA1AluB    AGGGGGATCAC--GAGGTCAGGAGTTCGAGACCAGCCTGACCAACATGGTGAAATCCCCG
SAA3Alu     GGGCAGATCACCTGAGGTCAAGAGTTCGAGACCAGCCTGGCCAACATGGCGAAATCC-TG
ConsAlu     GGGCGGATCACCTGAGGTCAGGAGTTCGAGACCAGCCTGGCCAACATGGTGAAA-CCCCG
             *    *****  ******* ******  ** ******* ********* **** **  *

SAA1AluA    TCTCTACTAAAAATACTAAAATGTAGCCAGG-CGTGGTGGCAGGCACCTGTAATCCCAGC
SAA2Alu     TGTCTACTAAAAATATGAAAATGTAGCCAGG-CGTGGTGGCAGGCAC-TGTAATCCCAGC
SAA1AluB    TCTCTACTAAAAATACAAAAAT-TAGCCAGG-CGTGATGGCGCATGCCTGTAATCTCAGC
SAA3Alu     TCTCTACTAAAAATACAGAAAT-TAGCCGGGTCGTCGTGGCATGCGCCTATAATCCCAGC
ConsAlu     TCTCTACTAAAAATACAAAAAT-TAGCCGGG-CGTGGTGGCGCGCGCCTGTAATCCCAGC
            * *************   **** ***** ** ***  ****     * * ***** ****

SAA1AluA    TACTCAGGAGGCTGA--AGCACGAGAATCACTTCAACCCGAGAAGCAGAGGTTGCAGTGA
SAA2Alu     TACTCAGGAGGCTGA--AGCAGGAAAAT-ACTTGAACC-GGGAAGCGGAAGTTGTAGTGA
SAA1AluB    TACTCAGGAGGCTGATCGGAAGAAGAATCTCTTGAACCTGG-AGGTGGAGGTTGCGATGA
SAA3Alu     CTCTCGGGAGGCTGA--GGGAGAAGAATTGCTTGAACTCAGGAGGCAGAGGTTGCAGTGA
ConsAlu     TACTCGGGAGGCTGA--GGCAGGAGAATCGCTTGAACCCGGGAGGCGGAGGTTGCAGTGA
              *** *********   * *  * ***  *** ***     * *  ** ****   ***

SAA1AluA    CTAGAGATCGCACCAGTGTC-CTCCAACCTGGGTGACAGA---GCGAGACT--CCATCTA
SAA2Alu     GTT-ACATTGCTCCAGTGTG-CTCCA-CCTGG-TGACAGAAGTGCAAGACATGCCGTCTA
SAA1AluB    GCTGAGATAGCACCACTGCAACTCCAGCCTGGGCGACAGAA---CTTTAGT--A-GTCAG
SAA3Alu     GCTGTGATCACACCACTGC-ACTCCAGCCTAGGAGACAGA---GCAAGACT--CCATCTA
ConsAlu     GCCGAGATCGCGCCACTGCA-CTCCAGCCTGGGCGACAGAG---CGAGACT--CCGTCTC
                  **  * *** **   ***** *** *  ******    *   *       **

SAA1AluA    AAAAAAATGAAAAAGAAAGTG-----CAGCCAAGGC
SAA2Alu     AAAAAATAAAAAAAGAAAAAAAAAGTCAACCACGGC
SAA1AluB    GGCAGAAG
SAA3Alu     GAGAGACAGAGAGAGAGAGAGAGGGAGAAATATACCCC
ConsAlu     AAAAAAAA
               * *
```

Figure 3.15 <u>Alignment of Human SAA Gene Intron 2 *Alu* Elements.</u>
The *Alu* elements including flanking repeats are shown aligned with the consensus sequence of Jurka and Smith (1988). Identity between all the sequences is indicated by an asterisk. The poly(A) sequences of the left and right *Alu* monomers can be seen at the beginning of the third and last blocks of sequence.

from man, mouse, monkey, Syrian hamster, mink, horse, Pekin duck, cow, cat and dog were aligned using the sequence alignment program Clustal (fig. 3.16A). Only gene products from individual gene loci and not alleles were used together with the leader sequence, where these parameters were known. Examination of the sequences showed strong cross-species conservation, particularly between residues 32 and 55 (relative to the mature huSAA1 peptide) 19 of which were entirely conserved, the remaining 5 having substitutions which maintained the same charge with the exception of residue 46 where both basic (K and R) and neutral (Q) amino acids were present. Interestingly this conserved region lies entirely within exon 3 of the human, mouse and hamster sequences. The sequence alignment also revealed two classes of proteins dependent on the presence or absence of an insertion between residues 69 and 70 relative to the human protein. Mink, horse, cat and dog proteins all contain an extra 8 amino acids in this region whereas bovine AA has 9 extra residues. Pekin duck AA defines another variant having a single extra amino acid in the same region. The underlying evolutionary mechanisms and the functional consequences of these differences is unknown. This area is within exon 4 of the human and murine genes and thus is unlikely to represent a splicing variant. The insertion occurs in a region which defines the end of a predicted ß hairpin and the start of an α-helical region in SAA1.

It has been proposed that the first 11 amino acids of the mature SAA proteins are involved in binding to the HDL particle based on the hydrophobic nature of the sequence in this region (Turnell *et al*, 1986). It can be seen that the human and murine SAA3 proteins (which have not been found in HDL) are diverged in this region compared to their respective SAA1 and SAA2 sequences which are found in HDL. However the SAA3 proteins are still predicted to be hydrophobic in this region, hence this may not account for the lack of association with HDL and other factors such as the site of production maybe responsible.

In order to give a graphical representation of the relative conservation of amino acid sequences the PROTPARS program of PHYLIP was run using 14 of the sequences from the alignment. The program calculates the minimum number of changes required to evolve from each of the sequences to each of the others and presents the results in the form of the most parsimonious tree (Felsenstein, 1990). Multiple runs of the analysis were carried out using different orders of species input as well as using the "jumble" option with various random number seeds to reduce the inherent bias in the program. An unrooted tree requiring the least number of steps is shown in figure 3.16B. In human, mouse, hamster and mink the SAA1 and SAA2 sequences are highly related, although less homologous in mink than in the other species. The SAA3 sequences where known are less homologous to SAA1 and SAA2 within the same species. Of note was the particularly strong homology between mouse and hamster SAA3, human SAA3 being less related to these but closer to the murine SAA1 and SAA2 sequences in structure than to the human sequences. The sequence from monkey AA was closely related to human

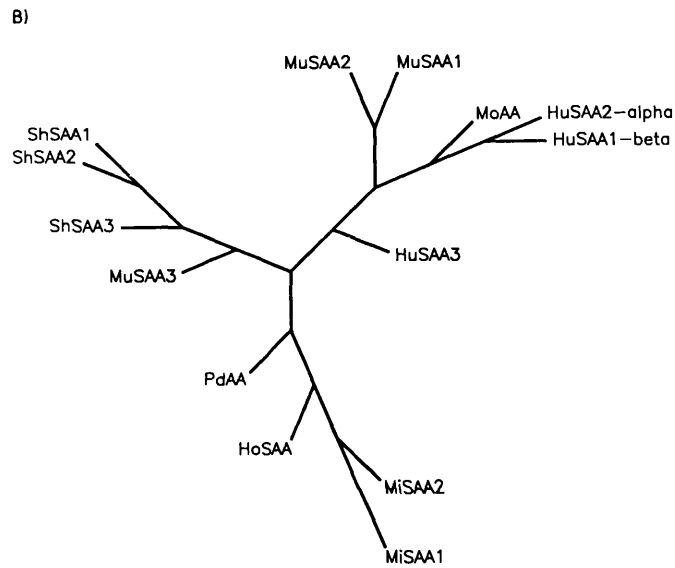
A)

```
          -18      -10        1         10         20         30         40
HuSAA1ß   MKLLTGLVFCSLVLGVSSRSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNYDAAKRG
HuSAA2α   MKLLTGLVFCSLVLGVSSRSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNYDAAKRG
HuSAA3    MKLSTGIIFCSLVLGVSSQGWLTFLKAAGQGAKDMWRAYSDMKEANYKKSDKYFHARGNYDAVQRG
MuSAA1    MKLLTSLVFCSLLLGVCHGGFFSFVHEAFQGAGDMWRAYTDMKEANWKNSDKYFHARGNYDAAQRG
MuSAA2    MKLLTSLVFCSLLLGVCHGGFFSFIGEAFQGAGDMWRAYTDMKEAGWKDGDKYFHARGNYDAAQRG
MuSAA3    MKPSIAIILCILILGVDSQRWVQFMKEAGQGSRDMWRAYSDMKKANWKNSDKYFHARGNYDAARRG
ShSAA1    MKPFVAIIFCFLVLGVDSQRWFQFMKEAGQGTRDMWRAYTDMREANWKNSDKYFHARGNYDAAQRG
ShSAA2    MKPFVSIIFCFLVLGVDSQRWFQFMKEAGQGTRDMWRAYTDMREANWKNSDKYFHARGNYDAAQRG
ShSAA3    MKPFLAIIFCFLILGVDSQRWFQFMKEAGQGSRDMWRAYSDMREANWKNSDKYFHARGNYDAAKRG
MiSAA1    MKLFTGLIFCSLVLGVSSQ-WYSFIGEAAQGAWDMYRAYSDMIEAKYKNSDKYFHARGNYDAAQRG
MiSAA2    MKLFTGLIFCSLVLGVSSQ-WYSFIGEAVQGAWDMYRAYSDMREANYKNSDKYFHARGNYDAAQRG
HoSAA                        LLSFLGEAARGTWDMLRATNDMREANYIGADKYFHARGNYDAAKRG
MoAA                       RSWFSFLGEAYDGARDMWRAYSDMKEANYKNSDKYFHARGNYDAAQRG
PdAA                    DNPFTRGGRFVLDAAGGAWDMLRAYRDMREANHIGADKYFHARGNYDAARRG
BoAA                         WMSFFGEAYEGAKDMWRAYSDMREANYKGADKYFHARGNYDAAQRG
CaAA                         WYSFVGEAAQGAWDMWRAYSDMREANYKNSDKYFHARGNYDAAQRG
FeAA                       EWYSFLGEAAQGAWDMWRAYSDMREANYIGADKYFHARGNYDAAQRG

           50         60                   70         80         90         100
HuSAA1ß   PGGAWAAEVISDARENIQRFF---------GHGAEDSLADQAANEWGRSGKDPNHFRPAGLPEKY
HuSAA2α   PGGAWAAEVISNARENIQRLT---------GHGAEDSLADQAANKWGRSGRDPNHFRPAGLPEKY
HuSAA3    PGGVWATEVISDARENVQRLT---------GDHAEDSLAGQATNKWGQSGKDPNHFRPAGLPEKY
MuSAA1    PGGAWAAEKISDGREAFQEFF---------GRGHEDTIADQEANRHGRSGKDPNYYRPPGLPDKY
MuSAA2    PGGVWAAEKISDARESFQEFF---------GRGHEDTMADQEANRHGRSGKDPNYYRPPGLPAKY
MuSAA3    PGGAWAAKVISDAREAVQKFT---------GHGAEDSRADQFANEWGRSGKDPNHFRPAGLPKRY
ShSAA1    PGGAWAAKVISDAREGFKRIT---------GRGIEDSRADQFANEWGRSGKDPNFFRPPGLPSKY
ShSAA2    PGGAWAAKVISDAREGFKRMR---------GRGIEDSRADQFANEWGRSGKDPNFFRPPGLPSKY
ShSAA3    PGGAWAAKVISDAREGIQRFT---------GRGAADSRADQFANKWGRSGKDPHHFRPAGLPSKY
MiSAA1    PGGAWAAKVISDARERSQRITD-LIKYGDSGHGVEDSKADQAANEWGRSGKDPNHFRPPGLPDKY
MiSAA2    PGGAWAAKVISDARERSQRVTD-LFKYGDSGHGVEDSKADQAANEWGRSGKDPNHFRPSGLPDKY
HoSAA     PGGAWAAKVISDARENFQRFTD-RFSFGGSGRGAEDSRADQAANEWGRSGKDPNHFRPHGLPDKY
MoAA      PGGVWAAEAISDARENIQKLL---------GHGAEDT
PdAA      PGGAWAARVISDARENWQG--------GVSGRGAEDTRADQEANAWGRNGGDPNRYRPPGLP
BoAA      PGGAWAAKVISDARENIQRFTDPLFKGTTSGQGQEDSRADQAA
CaAA      PGGAWAAKVISDARENSQRITD-LLRFGDSGHGAEDSKADQAANEWG
FeAA      PGGAWAAKVISDARENSQRVTD-FFRHGNSGHGAEDSKADQ---EWG
```

Figure 3.16 Phylogenetic Comparisons of SAA and AA Proteins.
A) The derived amino acid sequences for human SAA1ß and SAA2α were aligned with SAA3 and SAA and AA proteins of other species using the Clustal sequence alignment program. Allelic variants were not included. Numbering corresponds to the mature human SAA1 protein with residues identical among all species shown in bold. Dashes were introduced to maximize homology those between residues 69 and 70 represent the insertion found in a number of species. B) The most parsimonious (unrooted) tree created using the 14 uppermost sequences of the alignment shown in (A) with the PROTPARS program of PHYLIP. Bo=bovine (Benson *et al.*, 1989); Ca=canine, Fe=feline (Kluve-Beckerman *et al.*, 1989); Ho=horse (Sletten *et al.*, 1989); Hu=human (present report and Sack and Talbot, 1989); Mi=mink (Marhaug *et al.*, 1990); Mo=monkey (Hermodsen *et al.*, 1972); Mu=Balb/c mouse (Lowell *et al.*, 1986a); Pd=Pekin duck (Ericsson *et al.*, 1987); Sh=Syrian hamster (Webb *et al.*, 1989).

Figure 3.16B.


B)
MuSAA2
MuSAA1
MoAA
HuSAA2–alpha
HuSAA1–beta
ShSAA1
ShSAA2
ShSAA3
HuSAA3
MuSAA3
PdAA
HoSAA
MiSAA2
MiSAA1

SAA1 and SAA2 indicating that it was likely to be a product of the equivalent genes in the monkey. This strong homology was not unexpected considering the close evulotionary status of man and monkey.

In summary the data obtained for the human SAA genes in this study demonstrate the conservation of gene family structure between species both at the genomic level and in the encoded proteins.

CHAPTER 4.

Discussion of Gene Family Studies.

4.1 Human SAA is Encoded by a Small Multigene Family.

In order to determine the complexity of the human SAA gene family Southern analysis together with screening of genomic libraries was carried out. This established that the human SAA gene family comprises four members, three of which were cloned here: *SAA1ß, SAA2α* and *SAA4*. Together with *SAA3* (Sack and Talbot, 1989) and alleles at the *SAA1* and *SAA2* loci all the bands detected on Southerns in a Caucasian population could be accounted for. Research published prior to this study had established that a number of SAA genes and gene products were present in humans. A number of protein sequences together with the cDNA sequences of SAA1α and SAA2α (Sipe *et al*, 1985, 1986; Kluve-Beckerman *et al*, 1986a) had been determined. A genomic clone encoding SAA2ß had been characterized (Woo *et al*, 1987) and a number of partially characterized clones isolated (Sack, 1983). The results from these studies were entirely compatible with the work presented here and the existence of 4 SAA gene loci.

The results from Southern analysis were corroborated by those from four other published studies in the pattern of hybridizing fragments observed. Although some variability was evident in calculated fragment sizes from different studies, all the constant bands obtained can be assigned to the four SAA gene loci. This analysis is presented in table 4.1 in comparison with results from the present study. In the other studies it is appears that the hybridization stringencies used were higher thus the *SAA4* locus was not detected. Also under the conditions used by Steinkasserer *et al* (1990) the *SAA3* locus was not detected using probes equivalent to pA1a and pA1b (pAS_4 and pAS_1 respectively). However *SAA3* was detected in some of the digests (BglII with pAS_1, TaqI with pAS_4 and pAS_1, and EcoRI with pAS_1) this may reflect complete exon sequences hybridizing within fragments, hybridization to smaller fragments cleaved within exons being more difficult to detect in other digests. In the current work *SAA1* probe hybridized to *SAA3* and *SAA4*-containing fragments was removed when higher stringency washings were employed. Similar phenomena are also evident for many other gene families, for example genes related to the retinoic acid receptor homologues RXR (Mangelsdorf *et al*, 1990).

The *SAA3* probe used by Sack *et al* (1989) and Shohat *et al* (1990) hybridized strongly to the fragments assigned to *SAA3* (figure 3.13) hence confirming that, for example, the 3.6kb HindIII band represented *SAA3*. The use of a probe which contained intron 2 sequences (probe II, Sack *et al*, 1989) also confirmed that the weakly hybridizing bands between 1.5 and 2.3 kb, detected with pA1a, were fragments containing the 3' end of exon 2 and intron 2 of *SAA1* and *SAA2*.

The situation defined for humans was also comparable with that for the mouse where four gene loci are also present. Hybridization of probes that recognized *mSAA1, 2*

ENZYME	HindIII					BglII						PstI			
PROBE	pA1	I	II	pRI	82	pA1	I	II	pRI	4	1	a	b	I	II
SAA1	1.3	1.3	1.3 2.3*	1.3	1.1	4.4 0.7	4.5	4.5	4.5	4.5	4.5 0.7	1.2 1.9#	2.1	2.3	1.4
SAA2	4.6 /3.0	4.8 /3.0	4.8 /3.0 1.8*	4.3 /2.4	4.6 /2.8	15.0	9.0	9.0	16.5	12.5	12.5	2.7 /0.9 1.9#	2.7 /0.9	3.2 /1.8	3.2
SAA3	3.6	3.7	3.7	3.2	3.6	6.1	6.0	ND	6.5	ND	6.0	0.6	2.8	3.3 0.6	ND
SAA4	10.5	ND	ND	ND	ND	7.5	ND	ND	ND	ND	ND	1.8	2.5	ND	ND

ENZYME	PstI				TaqI						EcoRI				
PROBE	pRI	82	4	1	a	b	I	II	4	1	a	b	82	4	1
SAA1	1.3 2.1#	1.2 1.7#	1.4 1.8#	2.3	2.1 6.5	2.1	2.1	2.1	2.0 5.9	2.0	14.0	14.0	12.5	11.0	11.0
SAA2	2.6 /1.9	2.7 /0.8 1.9 1.7#	3.0 1.8#	3.0	4.3	4.3	4.8	4.8	4.0	4.0	15.0	15.0	14.7	11.5	11.5
SAA3	2.7 0.6	2.7	ND	ND	6.0	6.0	6.6	ND	5.7 /3.0	5.7 /3.0	1.4 17.0	1.4	ND	ND	1.7
SAA4	ND	ND	ND	ND	4.3	4.3	ND	ND	ND	ND	>20	>20	ND	ND	ND

Table 4.1 **Assignment of Bands in Published Southern Analyses to SAA Loci.**
The bands detected with various SAA probes by Southern analysis were assigned to the characterised loci. Probes "a" and "b" are pA1a and pA1b used in the present study; probe "I" = *SAA3* exon 3+4; "II" = *SAA2ß* 3' intron 2 + exon 3 (Sack *et al.*, 1989); "pRI" = *SAA3* probe (Shohat *et al.*, 1990); pAS_4 and pAS_1 are cDNA probes equivalent to pA1a and pA1b (Steinkasserer *et al.*, 1990); pSAA82 = SAA2α cDNA probe (Kluve-Beckerman *et al.*, 1986a). * These extra bands are also detected weakly with pA1a and represent exon 2 hybridization (see fig. 3.1); # fragments containing exons 1 and 2 of *SAA1* and *SAA2*; / = allelic fragments. Extra polymorphic bands were detected with pAS_4 and pAS_1 at 4.9kb for PstI in 2 individuals and a 5.5kb TaqI fragment with pAS_1 in one individual.

and *3* gave multiple bands, for example, three fragments for HindIII, BglII and PstI with Balb/c mouse DNA (Lowell *et al*, 1986a).

Steinkasserer *et al* (1990) cloned five distinct cDNAs from a single individual which can be equated to three gene loci. However an *SAA3*-derived product was not obtained implying the existence of a further expressing locus, a finding incompatible with the results presented here. Three cDNAs were products of the *SAA1*α, *SAA2*α and *SAA2*β genes, but two clones, $pAS_{3\alpha}$ and $pAS_{3\beta}$ were identical to *SAA1*α and *SAA1*β respectively including the 3'-untranslated region with the exception of a single nucleotide substitution at codon 60 (G to A) causing an Asp to Asn coding difference. This was unlikely to be an *SAA4* gene product for two main reasons, firstly as shown by the Southern and cloning data *SAA4* does not show strong homology to *SAA1* whereas pAS_3 does. Secondly an *SAA1*-specific oligonucleotide which recognizes both *SAA1* and pAS_3 sequences due to their homology hybridized to a single 11kb EcoRI fragment (Steinkasserer *et al*, 1990) whereas *SAA4* was present on a >20kb EcoRI fragment. The pAS_3 gene was not present within the same EcoRI fragment as *SAA1* as shown by the λ1.1, 1.2 and 1.3 clones (figure 3.12) whose overlap was confirmed by restriction mapping and sequence identity. Potentially the *SAA1* and pAS_3 genes could be present on the same size fragments in all the digests examined by Southern analysis, however the hybridization intensity of *SAA1*-containing fragments (quantitated by densitometric scanning using *SAA2* bands as an internal marker) was compatible with a single gene. Finally the three *SAA1* BglI alleles were inherited in a Mendelian fashion, if a second non-allelic *SAA1*-paralogous gene had been present Mendelian inheritance would not have been demonstrable. Thus it appears likely that a duplication occurred within the parental population of the individual from which the cDNA library was made. The Southerns presented in the report of Steinkasserer *et al* (1990) were compatible with the previous data (table 4.1) although this analysis was not available for the individual from which the cDNA library was made. Gene dosage polymorphisms have been observed between populations in a number of gene families possibly arising by mechanisms such as those described in section 1.4. The human α-amylase gene family provides such an example with heterogeneity possibly caused by homologous unequal cross-over events (Groot *et al*, 1989). Duplications of SAA genes have been recorded in wild mice (Yamamoto *et al*, 1988). We also have preliminary evidence that in humans duplications occur in rare cases (see figure 3.1 where DNA from the same individual is present in lanes 3 of c and g, compared to the *SAA1* bands of the other individuals). The G/A difference between *SAA1* and pAS_3 occurs within a CpG dinucleotide, hence a C to T transformation on the lower strand within the complementary CpG would create an A on the upper strand. As described previously CpGs are mutational hotspots in the genome with deamination of 5mC to T (Bird, *et al*, 1987), thus the possibility that the difference in the SAA sequences was caused by an RNA processing mechanism seems remote.

RFLPs were detected in a number of studies in the SAA genes including the *SAA2* HindIII and PstI polymorphisms (Kluve-Beckerman *et al*, 1986b; Sack *et al*, 1988; Shohat *et al*, 1990). An additional 4.9kb PstI fragment was found by Steinkasserer *et al* (1990) which has not been detected elsewhere and at present the locus from which it was derived is unknown. Thus together with the NcoI RFLP the *SAA2* locus appears to be highly polymorphic. The BglI polymorphism was the first one discovered for the *SAA1* locus where the largest allele was represented by the genomic clone obtained. Interestingly the cDNA clone pAS_1 (Steinkasserer *et al*, 1990) represents one of the smaller two alleles with a single nucleotide difference at codon 78 creating a BglI site. The nature of the third allele is unknown but could possibly be determined by PCR amplification of the *SAA1* locus from individuals with a 2,2 or 3,3 genotype, with subsequent digestion with BglI. A *SAA1* polymorphism was detected by Shohat *et al* (1990) with TaqI but was not seen in 30 individuals studied here. Another TaqI RFLP found by the same workers for the *SAA3* gene produced a 9.5kb fragment not found here, however the 2.9kb *SAA3* TaqI polymorphic band (which may be the same as the 3.0 kb fragment of Steinkasserer *et al*, 1990) was present in one of thirty individuals. The frequency of this TaqI allele in Armenians was 0.015 (Shohat *et al*, 1990) and would appear to have a similarly low frequency in Caucasians. Thus polymorphisms have now been detected in all but one of the SAA gene loci. The SAA cloning data from a number of studies is summarized in table 4.2.

Table 4.2 Genomic and cDNA Clones Obtained for Human SAA.

	Genomic clones	cDNA clones
1) SAA1α	-	pA1, pAS_1, ($pAS_{3\alpha}$)
2) SAA1ß	λ1.1	($pAS_{3\beta}$)
3) SAA2α	λ2.2, GSAA3	pA10, pSAA82, pAS_8
4) SAA2ß	λ2.1, cos2.3	pAS_2
5) SAA3	GSAA1	
6) SAA4	cos2.3	

Genomic clones are shown with their corresponding cDNAs and proteins. The pAS_3 clones are shown in brackets as they showed near identity to SAA1 but maybe the products of a duplicated locus. Data were from the current study and Sipe *et al* (1985); Kluve-Beckerman *et al*, (1986); Sack and Talbot (1989) and Steinkasserer *et al*, (1990).

4.2 Phylogeny and Evolution of SAA Genes.

The human SAA gene family shows striking homology to the murine equivalent. Both comprise four discrete loci with two highly related genes (*SAA1* and *SAA2*) and two genes which have diverged from these. In the mouse *SAA4* is a pseudogene as 5' sequences have been deleted. At present it is not known whether human *SAA4* is also a

pseudogene although exon 2 hybridizing sequences were not detected with pA1a indicating either a diverged or deleted 5' portion. The murine genes are present at a single locus on a 79kb region of chromosome 7. The human genes have been mapped to 11p11 to 11pter using somatic cell hybrids (Kluve-Beckerman *et al*, 1986b). This is compatible with the synteny between these chromosomes in humans and mice. We also have evidence that the human genes are present in a single chromosomal region from pulsed field mapping data. Using high molecular weight human DNA immobilized in agarose blocks, restriction digests were carried out with a number of rare cutting enzymes and fragments separated by crossed field gel electrophoresis (Southern *et al*, 1987). The results of such an analysis are shown in figure 4.1. pA1 hybridized to two MluI fragments (250 and 450kb) and single NotI (350kb) and BssHII (405kb) fragments. Thus the SAA genes appear to be within a 350kb region as defined by the NotI digest. However hybridization of gene-specific probes for all four human genes to this fragment will have to be demonstrated. Multiple rare-cutter enzyme digests will allow further definition of the smallest region containing all four genes. The use of the other chromosome 11 probes will enable linkage to other loci to be established using a combination of pulsed field and genetic analyses. In this respect the RFLPs identified will be useful for genetic mapping. Experiments are currently in progress to further localize the SAA genes using a panel of human/rodent somatic cell hybrids containing deletions or translocations of 11p segments.

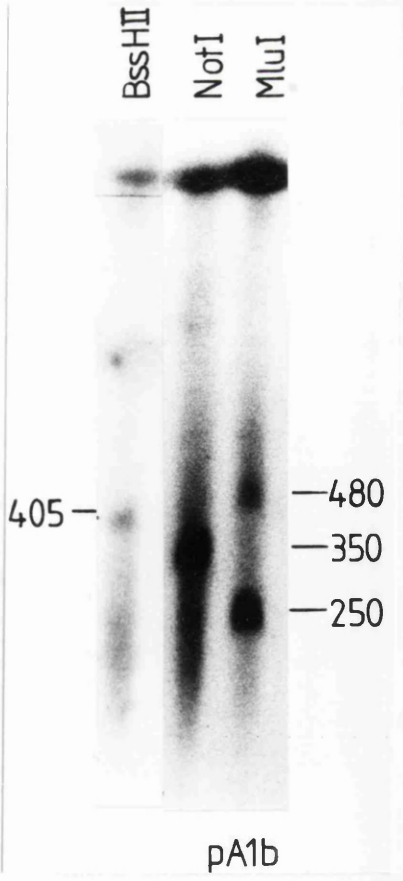


Figure 4.1 PFGE Analysis of Human Genomic DNA with a SAA Probe.
Large molecular weight DNA digested with the enzymes shown was separated by CFGE, blotted and probed with pA1b. Estimated fragment sizes of the hybridizing bands are shown in kb.

The exon/intron structures of the human genes are also similar to those of the mouse. In both species *SAA3* has a larger first and second intron but a smaller third intron compared to *SAA1/2* which have similar spacings (compare figure 3.12 and figure 1 in Lowell *et al*, 1986a). However in humans *SAA1* intron 2 is 300 bp longer than that of *SAA2* due to the presence of an extra *Alu* repeat (*SAA1AluB*). In the mouse *SAA1* and *SAA2* are found as apoproteins of HDL; this is also true of humans where six plasma isoforms (Bausserman *et al*, 1980) can be equated to *SAA1* and *SAA2* loci (Strachan *et al*, 1989). In contrast SAA3 in both species has not been found associated with HDL and in humans no gene product has been identified at the mRNA or protein levels. In terms of amyloid deposition only AA derived from *SAA2* is found in murine deposits (Shiroo *et al*, 1987). This is thought to be due to differing secondary strucures, as despite strong primary sequence homology, SAA1 is predicted to have twice the α-helical content of SAA2 and a lower propensity to aggregate (McCubbin *et al*, 1988). This association cannot be extrapolated to humans where both SAA1- and SAA2-derived molecules are found in amyloid (see table 4.3 and references in legend).

An evolutionary model has been proposed for the murine SAA genes in which an ancestral SAA gene duplicated to create a proto-*SAA1/SAA2* gene and *SAA3* which then diverged. At a later date the proto-*SAA1/SAA2* gene duplicated to form *SAA1* and *SAA2* whose high degree of homology has been maintained since by one or more gene conversion events (Lowell *et al*, 1986a; Yamamoto *et al*, 1987). The analogy between the human and murine gene structures suggests that a similar model maybe applicable to humans. The structural homology between the two species indicates that the current organization may have been determined prior to divergence of human and rodent lineages. Gene conversion is a possible mechanism by which *SAA1/SAA2* homology has been maintained over this time. However *SAA1* and *SAA2* sequences could potentially become homogenized by "illegitimate" recombination between *SAA1* and *SAA2* genes on paired chromatids at meiosis. This would have to occur via double homologous recombination otherwise expansion/ contraction in SAA gene number would occur. The double cross-over event is unlikely due to the lack of homology between the *SAA1* and *SAA2* genes outside the immediate 5' and 3' flanking regions. This does not rule out a role for recombination as it has been proposed that gene conversion can occur as an aborted recombination event or *en route* to recombination (Kourilsky, 1986), gene conversion being able to occur intra- or extra-chromosomally (between sister chromatids). Regions of homology occur 5' of the *hSAA1/2* homology unit (i.e. upstream of -450bp, see section 5.2) which would not be predicted in a gene conversion model, however this is not unprecedented. For example the foetal globin genes $^{G}\gamma$ and $^{A}\gamma$ are part of a large (5kb) duplication unit much of which has diverged by 10-20%, however a 1.5kb region of virtually identical sequence is involved in intergenic events (Slightom *et al*, 1980). The foetal γ-globin gene conversions are limited to transcriptional domains, 5' regulatory sequences and extending to just beyond the poly(A) signal (Fitch *et al*, 1990) a situation

which is clearly analogous to *hSAA1/SAA2*. Potential molecular events in the evolution of the human SAA gene family are shown in figure 4.2.

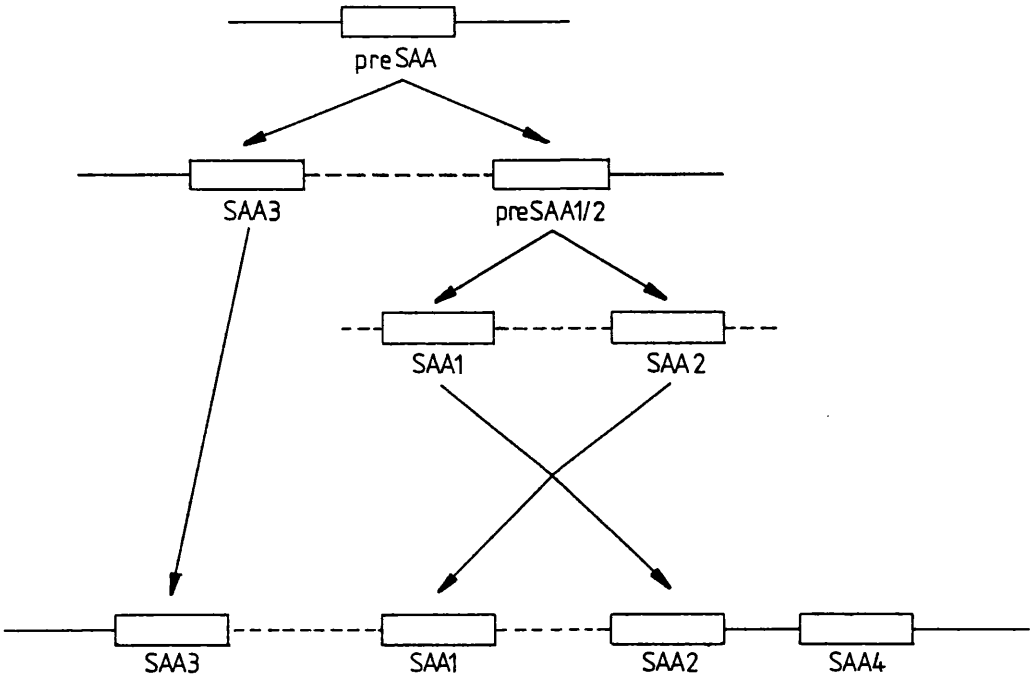


Figure 4.2 Evolution of the Human SAA Genes.
Potential steps in the evolution of SAA genes are shown starting from an ancestral sequence which duplicated possibly via unequal homologous cross-over between flanking repetitive sequences. The genes could then evolve further by homologous unequal cross-over between *SAA3* and pre*SAA1/2* or via repeats flanking *SAA1/2*. *SAA1* and *SAA2* sequences underwent concerted evolution possibly by gene conversion (shown by converging arrows) with *SAA3* evolving independently. At some stage *SAA4* also arose maybe involving duplication of DNA sequences or retroposition. The orientations and distances between the sequences are arbitrary (except *SAA2* and *SAA4*). However pulsed-field mapping data indicates that the genes are within the same chromosomal region.

Repetitive elements have been found at the termini of gene conversion units (Fitch *et al*, 1990) and CA/GT repeats were found 5' of *mSAA1* and *mSAA2* (Lowell *et al*, 1986a; Yamamoto *et al*, 1987). The 5' cut-off point of homology between the human *SAA1* and *SAA2* genes is not so well defined due to the regions of homology upstream of -450bp. However a polypurine/ polypyrimidine tract is found upstream of -800bp in *SAA1* which may potentially be involved in gene conversion. Other sequence elements may have a role in promoting gene conversion. Jeffreys *et al* (1985) noted the similarity between a minisatellite core sequence and the cross-over hotspot instigator (Chi) sequence of *E.coli*. Chi functions as a signal for homologous recombination (Smith,

Protein	RESIDUE -4	15	23	25	30	31	45	46	52	53	55	57	58	60	65	66	68	69	71	72	75	79	82	84	87	90	Ref.
SAA1α	G	R	D	R	I	G	A	K	V	W	A	A	I	D	I	Q	F	F	H	G	D	D	A	E	R	K	[1]
SAA1β									A			V															[2]
pAS3α														N													[3]
pAS3β									A			V		N													[3]
SAA2α	S								A			V		N			L	T						K		R	[4]
SAA2β	S								A			V		N			L	T	R					K		R	[5]
SAA2β									A			V		N			L	T	R					K		R	[6]
SAA3		K		K	K	K	V	Q			T	V			V		L	T	D	H		G	T	K	Q		[7]
SAA1α/β									V/A			A/V															[8]
SAA2α/β									A			V		N			L	T	H/R					K		R	[9]
SAA			N						V/A			A/VI/L		N													[10]
AA			N						V/AW/R							E					N	\\\\\\\\\\\\\\				[11]
AA									A W/R			V		N			L	T	R			\\\\\\\\\\\\\\					[12]
AA			N																		N	\\\\\\\\\\\\\\					[13]

Table 4.3 Variable Amino Acid Residues Found in Human SAA/AA Proteins.
SAA1α amino acids are shown at those residues known to be polymorphic in humans. The upper six sequences are deduced from cDNA and genomic cloning data, the lower sequences were determined by direct sequencing of isolated proteins. Residues identical to SAA1 are shown blank. References: 1, Sipe *et al.* (1985), Kluve-Beckerman *et al.* (1988); 2, present study; 3, Steinkasserer *et al.* (1990) 4, Kluve-Beckerman *et al.* (1988), present study; 5, Kluve-Beckerman *et al.* (1986, 1988); 6, Woo *et al.* (1987); 7, Sack and Talbot (1989); 8, Parmelee *et al.* (1982); 9, Dwulet *et al.* (1988), Kluve-Beckerman *et al.* (1988); 10, Sletten *et al.* (1983); 11, Moyner *et al.* (1980); 12, Levin, *et al.* (1972); 13, Sletten and Husby (1974).

1987) by binding RecBCD which unwinds and nicks the DNA to create a free single strand that can invade an homologous duplex to form a Holliday intermediate. It was suggested that the minisatellite core sequences may be involved in creating length hypervariability (see Jarman and Wells, 1989). In this "recombinator hypothesis" the duplex is unwound by recombination enzymes recognizing the minisatellite core sequence followed by nicking to give a single strand projection. The production of such single strand breaks could lead to invasion of an homologous duplex and subsequent gene conversion (see figure 1.2). Homologies to Chi sequences are found in intron 3 of *SAA1* and *SAA2* (see figure 3.8) and thus could be initiation sites for conversion. Support for support for a role of minisatellites sequences in recombination in eukaryotes is provided by the presence of such sequences in the recombination hotspot of the human IgH locus (Keyeux *et al*, 1989).

Irrespective of the actual model for the occurrence of strong homology, *hSAA1* and *hSAA2* are clearly more related to each other than to the orthologous genes in the mouse. This would not be expected in the absence of gene conversion (Wu and Li, 1985) assuming duplication occurred prior to speciation.

The human genome has a number of classes of repetitive elements dispersed throughout it, these include the short and long interspersed repetitive elements (SINEs and LINEs). Both are characterized by flanking direct repeats and are 3' A-rich, hence are probably formed by retroposition. *Alu* repeats are the best characterized SINEs comprizing a left and right monomer related to the RNA polIII transcribed 7SL-RNA a component of the signal recognition particle (Walter and Blobel, 1982; reveiwed by Schmid and Jelinek, 1982). Over $5x10^5$ of these repeats are dispersed throughout the human genome (Hwu *et al*, 1986). The second introns of *hSAA1-3* all contain *Alu* family repeat sequences belonging to the "Sa" subclass of Alu repeats (Jurka and Smith, 1988). In addition each was flanked by direct repeats which fitted the classification of Daniels and Deininger (1985) in being dA-rich at their 5' ends (except *SAA1AluB*) and are presumably duplications of the insertion target site.

The presence of *Alu*s in *hSAA1-3* has implications for SAA gene evolution. The insertions could have occurred in the primordial gene prior to duplication or individually into each of the genes. If the former was the case similar elements would be expected within the murine SAA genes, although it has been reported that dispersion of *Alu* sequences occurred after human-mouse divergence (see Hastie, 1989). However when a mouse B1-element (i.e. 7SL-RNA-related sequence) probe was hybridized to the murine SAA clones a positive signal was found in *mSAA3* intron 2 (Lowell *et al*, 1986a). Potentially this could be an *Alu* repeat as 300bp *Alu* homologues have recently been found in the mouse genome (Hastie, 1989). Similar repeats do not appear in *mSAA1/2*, indicating their deletion in these genes or post-speciation insertion. The current assumptions that not all *Alu* repeats are sources of further retropositions (Labuda and Striker, 1989) but that certain "master" sequences are active throughout evolution (Britten

et al, 1988) suggests that the *Alu* repeats in the human *SAA* genes were inserted during the same evolutionary period. *SAA1AluB* was probably inserted after *SAA1/SAA2* duplication and possibly after the last gene conversion event although the regions 5' and 3' of *SAA1AluB* could act as independent gene conversion units. These findings indicate that in some way the SAA introns maybe insertion hotspots. In addition *Alu* repeats were also found in the 5'-flanking regions of both *SAA1* and *SAA3* (figure 3.14A) indicating that insertions maybe responsible for altering gene expression. The presence of multiple *Alu* repeats in the SAA locus raises the possibility that evolutionary alterations in gene structure may occur via recombination between repeats. For example *Alu-Alu* recombination in the LDL-R gene has resulted in deletion of exons encoding transmembrane and cytoplasmic portions in certain families (Lehrmann *et al*, 1985). Genetic variation in the human glycophorin gene family (Kudo *et al*, 1989) and the α-globin gene cluster (Nicholls *et al*, 1987) have arisen by similar mechanisms.

In addition to man and mouse there is evidence from cDNA and genomic cloning data for SAA gene families in hamster, mink, rat, cat and dog (Webb *et al*, 1989; Marhaug *et al*, 1990; Meek and Benditt, 1989; Harats *et al*, 1991; Sellar *et al*, 1991). Of these one Syrian hamster genomic clone has been obtained which has a similar exon/intron organization to the murine and human genes although exon 1 was not identified. Multiple hybridizing bands were obtained in Southern analysis of Syrian hamster genomic DNA and 2 cDNAs, distinct from the genomic clone were isolated (Webb *et al*, 1989). The existence of at least two genes in the mink is likely on consideration of cDNA cloning data (Marhaug *et al*, 1990). *mSAA1/2* and *mSAA3* related genes are also present in the rat although a deletion manifest in the *mSAA1/2*-related mRNA may be responsible for the lack of circulating SAA in the rat during the APR (Meek and Benditt, 1989). Reports at a recent meeting (Harats *et al*, 1991) also showed at least 3 genes were present in the Abyssinian cat. In the dog 5 distinct cDNAs which are probably the products of 3 gene loci have been cloned (Sellar *et al*, 1990). Thus it appears that many species of vertebrates have an SAA gene family. Further delineation of gene structures in different species will enable the proposed evolutionary model to be confirmed and refined.

At the protein level, the known SAA proteins from different species showed a high degree of homology (see section 3.2.7.2 and figure 3.16). This homology was particularly strong between residues 32 and 51, indicating structural and functional importance. Within this region the sequence Gly^{48}-Pro^{49}-Gly^{50}-Gly^{51} occurs, homologous to Gly-X-Gly-Gly found in all phospholipase A_2 sequences forming a calcium binding site (Turnell *et al*, 1986a). Amyloid deposits are rich in calcium and these ions may also be important in binding to lipid. Early structural analyses of human amyloid A revealed α-helical structure consisting of polar and non-polar faces with a regular charge distribution (amphipathic helix), a motif characteristic of lipid binding proteins (Segrest *et al*, 1976). AA was found to bind to lipid and the 26 N-terminal

residues fitted an α-amphipathic helical model. Parmelee *et al* (1982) later confirmed this data and proposed that residues 1-24 and 50-74 formed α-helices of about 7 turns each. The later analysis of Turnell *et al* (1986a) further delineated these regions into an α-amphipathic helix between residues 2 and 8 which forms two turns and could span the HDL lipid monolayer. Towards residue 12 the predicted hydrophilicity increased markedly leading to a decrease in amphipathic intensity. Other structural motifs included two ß-hairpins with α-helical structure between residues 12-27 and 72-86. (The predicted secondary structure of apoSAA1α is shown schematically in figure 5.3).

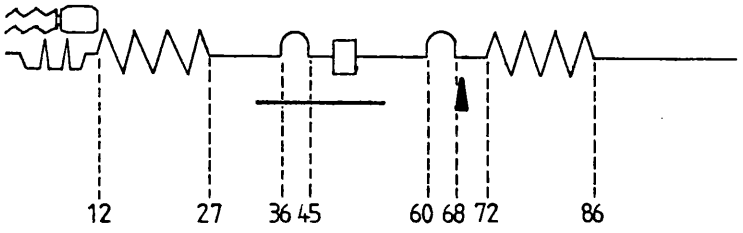


Figure 4.3 Schematic Representation of an SAA Molecule.
The predicted secondary structure for human apoSAA1α is shown using data from Turnell *et al*, (1986a). Structural domains shown linearly are, amino acids 1-12 α amphipathic helix adjacent to phospholipid molecule; 12-27 and 72-86 = α-helix; 36-45 and 60-68 = ß hairpin; 48-51 = calcium binding site (*boxed*); the remainder of the molecule forms random coils. The *horizontal bar* indicates the phylogenetically conserved region and the *upward arrowhead* the position of the 8 or 9 amino acid insertion occurring in SAA proteins of some species (see figure 3.16).

The formation of AA fibrils maybe brought about initially via dimerization through hydrogen-bonding between pairs of anti-parallel ß strands, dimers then interacting with each other via the hydrophobic regions of the molecules (Turnell *et al*, 1986b). All the SAA molecules from which AA is derived have these structural features.

Although hSAA3 and mSAA3 have not been found associated with HDL and have diverged N-terminal sequences compared to the respective SAA1 and SAA2 proteins, the linear pattern of hydrophilicity is maintained. This suggests that they maybe able to associate with lipid but that this is limited due to the site of production, other structural features or association with different lipid fractions. The predicted human SAA3 protein has homology to the rabbit synovial fibroblast-derived SAA-like collagenase inducer (Sack and Talbot, 1989) and if induced in the inflamed joint could play a role in connective tissue breakdown.

CHAPTER 5.

Transcriptional Studies of the Human SAA genes.

5.1 Introduction.

There are several potential mechanisms whereby the level or activity of a particular gene product can be controlled, from transcription of the gene to degradation of the protein. Whereas mRNA stability and secretion rates are altered for at least some of the acute phase proteins, transcription is the primary control point for their expression (see table 1.4). Inflammatory cytokines such as IL1 and IL6 have been shown to induce transcription of CRP (Ganter *et al*, 1989), Hx (Poli and Cortese, 1989) and Hp (Morrone *et al*, 1988). Transcriptional control has also been demonstrated to be important for both human (Edbrooke *et al*, 1989) and murine (Lowell *et al*, 1986b) SAA. In a number of cases inducible *trans*-acting factors which interact with acute phase gene promoters/enhancers have also been identified (*e.g.* Edbrooke *et al*, 1989; Majello *et al*, 1990; Nonaka and Huang, 1990; Ron *et al*, 1990).

Because of the stringent regulation of SAA and the amplitude of its induction in the liver during the APR, SAA genes represent a model system for the study of temporal and spatial control of gene expression. Having structurally characterized *SAA1* it was first necessary to determine whether this gene was inducible by inflammatory cytokines and to compare any such induction with *SAA2*. This was studied using *SAA/CAT* reporter genes transiently transfected into the human hepatoma cell line HepG2. When this work commenced the major area of study in this laboratory was the transcriptional control of SAA by IL1, thus the possible effects of other cytokines, particularly IL6 were also of interest. This was investigated using both functional studies with various *SAA/CAT* reporter genes and bandshifting/footprinting studies to assay interactions of nuclear proteins from cytokine treated HepG2 cells with SAA promoter fragments. *In vivo* the hepatocyte is exposed to a cocktail of cytokines and detailed accounts of the interactive nature in controlling acute phase genes had not been published. The combinatorial effects of cytokines were therefore investigated.

5.2 Results.

5.2.1 Potential Cis-Acting Elements in the SAA1 5' Flanking Region.

The sequences immediately upstream of *SAA1* exon 1 were analysed for homologies to known *cis*-acting regulatory elements, potentially involved in tissue-specificity or acute phase induction. The following matches to known consensus sequences were found, (see also figure 5.1),

i) a TATA box, 5'-TATAAAT-3' approximately 30bp upstream of exon 1, the binding site for the general transcription factor TFIID;

ii) a decamer sequence with homology to the binding site to the transcription factor NFκB, 5'-GGGACTTTCC-3' at -91 to -82 originally identified in the immunoglobulin κ light chain enhancer (Sen and Baltimore, 1986);

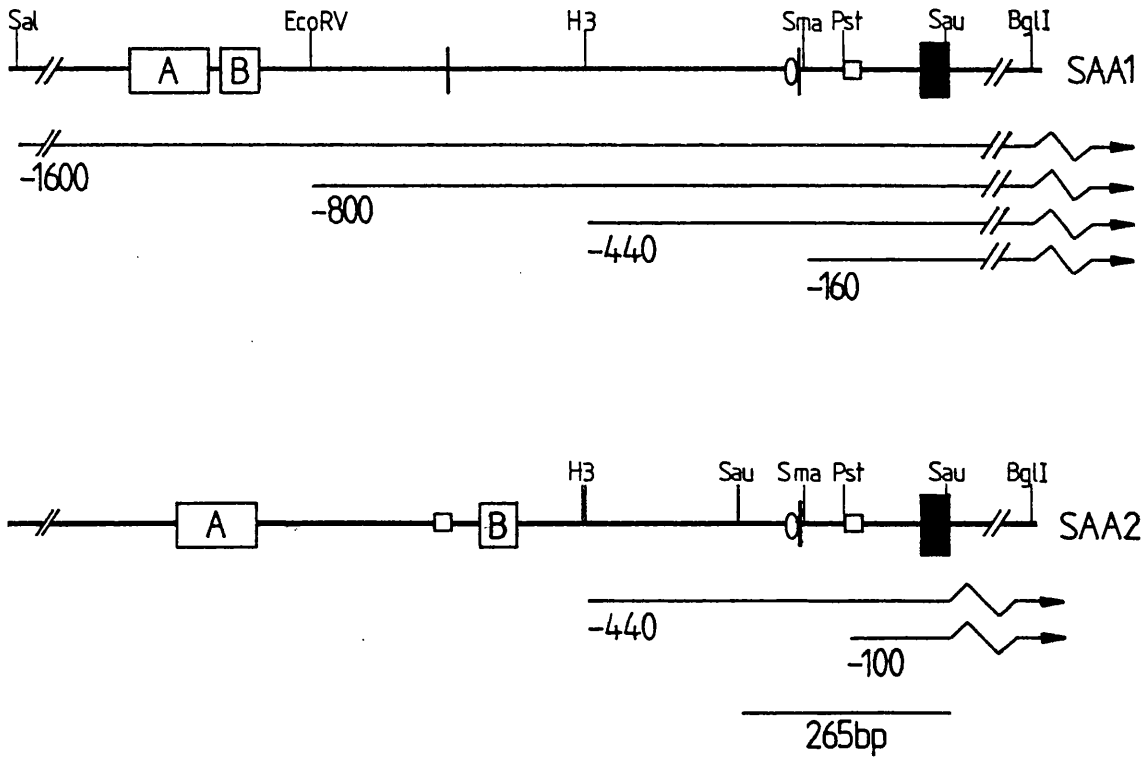


Figure 5.1 Comparison of the Structure of the *SAA1* and *SAA2* 5' Region and CAT Constructs.
The CAT constructs used in these studies are shown below representations of the *SAA1* and *SAA2* 5' regions. Solid box = exon 1, open boxes = NFκB sites, open circle = NF-IL6 homology, vertical bars = CTGGGA elements. The HindII sites (H3) shown represent the 5' limit of the *SAA1/SAA2* homology unit with the exception of elements A and B. The bar labelled 265bp indicates the *SAA2* Sau3A fragment used in bandshift studies.

iii) a hexanucleotide sequence 5'-CTGGGA-3' occured between nucleotides -167 and -162, this element has been found a large number of AP genes in a number of species (human, rat, mouse) and the possible involvement of this sequence and adjacent ones in the IL6 induction of rat α_2M, human haptoglobin and human CRP has been shown (Kunz *et al*, 1989; Oliviero and Cortese, 1989; Hattori *et al*, 1990; Majello *et al*, 1990). A second copy of this element was also present further upstream between -629 and -624;
iv) immediately 5' of the proximal CTGGGA element is an 8 nucleotide element which shows complete identity with the recently characterized NFIL6 binding site, namely 5'-TTGCACAA-3'. This regulatory sequence is involved in IL1 induction of the IL6 gene and homologous regions were found in a number of AP genes (Isshiki *et al*, 1990; Akira *et al*, 1990);
v) the region between -114 and -72 shows 67% homology to the equivalent region of the murine *SAA1* and *SAA2* genes (Lowell *et al*, 1986b) and thus maybe important in acute phase gene expression;
vi) a 17 out of 22 match to the IFNα consensus sequence occurs between -72 and -53.

These elements were also found in the same region of *SAA2* to which *SAA1* was 88% homologous up to -450bp. The homology breaksdown completely upstream of this due to an *Alu* repeat insertion with the exception of two regions between -1050 and -850 of *SAA1* (elements A and B, figure 5.1) which were 91% and 82% homologous to -977 to -873 and -586 to -535 of *SAA2*. Differences occurring in *SAA2* which maybe of significance include a single altered base in the NFIL6 consensus, the upstream CTGGGA was degenerate and finally *SAA1* does not contain a second NFκB site found at -635 to -626 in *SAA2* (SAAκB$_2$) which is in the opposite transcriptonal orientation to SAAκB$_1$. To investigate whether this sequence homology was representative of similar patterns of gene expression, transfection studies were carried out with *SAA1* and *SAA2* reporter genes.

5.2.2 Analysis of SAA gene Expression by Transient Transfection.

5.2.2.1 Reporter Gene Constructions.

Transient transfection of DNA constructs into mammalian cells allows the analysis of large numbers of reporter gene constructions with varying test sequences. Chloramphenicol acetyl transferase (CAT) constructs were made containing various lengths of sequence upstream of the first exon of *SAA1*. A 2200bp BamHI/BglI *SAA1* gene fragment containing 1600bp 5' of exon 1, exon 1 and 404bp of intron 1 was isolated from the Bluescript subclone KS7 (KS7= λ1.2 SalI/XhoI 2500bp fragment in Bluescript KS XhoI site). The BglI overhang was removed using the 3'-5' exonuclease activity of T4 DNA polymerase. The vector pTKCAT3 (Miksicek *et al*, 1986) containing the CAT gene was cut with BglII and the 5' overhang filled in using Klenow polymerase, a second restriction with BamHI removed the TK promoter and allowed the cloning of the *SAA1* BamHI/BglI(blunt) fragment. This construct, *SAA1CAT*1600 therefore contained -1600 to +404 of *SAA1* upstream of the *CAT* gene. This cloning strategy also resulted in the

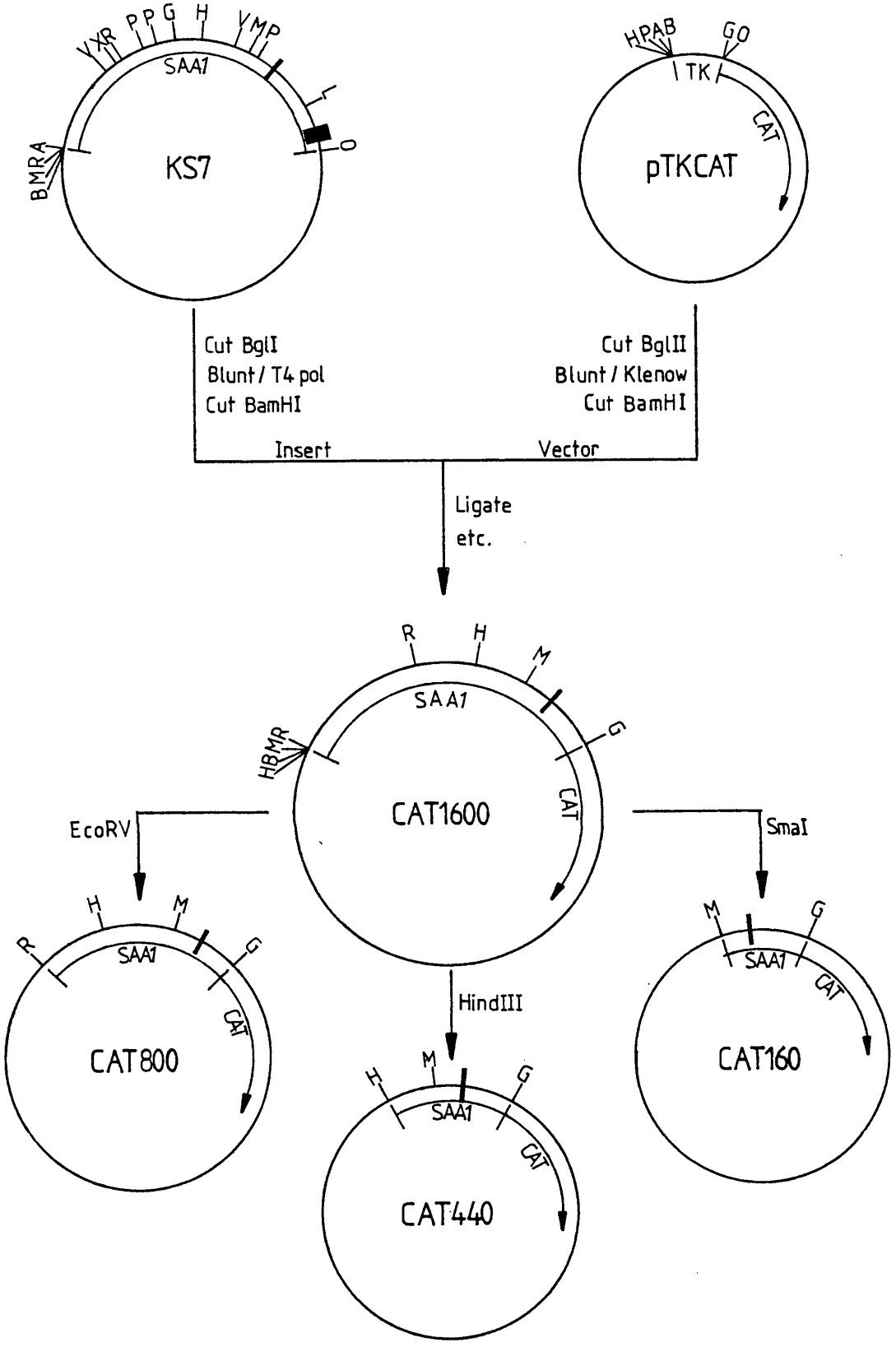


Figure 5.2 Construction of *SAA1/CAT* Fusion Genes.
The reporter gene constructs were made as described in the text. The complete restriction maps for the inserts of KS7 and TKCAT are shown whereas only those used in cloning are shown thereafter. Abbreviations for restriction sites are as chapter 3 but also include R=EcoRV, V=PvuII.

insertion of the 55bp BamHI to XhoI region of the Bluescript polylinker 5' to the SAA insert. The following deletion constructs were also made to:

i) -800bp by removing the 5' SmaI/EcoRV-3' 800bp fragment and religating;

ii) -440bp by restricting with HindIII and religating;

iii) -160bp by removing a 1440bp SmaI/SmaI fragment and religating (see fig. 5.2).

All constructs were grown up and checked by extensive restriction mapping and sequence analysis. All *SAA2CAT* genes used in these studies were constructed by other members of the laboratory, these contained various lengths of the *SAA2* promoter region 5' to the Sau3A site in exon 1 fused to *CAT* in the same vector (see fig. 5.1).

5.2.2.2 Analysis of SAA Gene Expression in Response to cytokines.

Following transfection of reporter gene constructs into HepG2 cells as described in section 2.2.2.2 cells were treated with rhIL1ß at $1Uml^{-1}$, rhIL6 at $100Uml^{-1}$ or rhTNFα at $100Uml^{-1}$ for 16 hours. The result obtained following stimulation with IL1ß and IL6 in a typical CAT assay are shown in figure 5.3. The acetylated and non-acetylated products were cut out and quantitated by liquid scintillation counting, the calculated percent conversion was then normalized for transfection efficiency. This analysis produces the results shown graphically in figure 5.4 where the result obtained for *SAA1CAT*440 is compared with that for *SAA2CAT*440. The mean ratios of induced:uninduced are shown, it can be see that $1Uml^{-1}$ IL1ß stimulated *SAA1CAT*440 3.8 fold and *SAA2CAT*440 3.7 fold, this compared to 2.4 and 2.3 fold stimulations respectively by $100Uml^{-1}$ of IL6 for these two constructs. However incubation with both cytokines concurrently at the same concentrations gave an approximately 20 fold elevation of CAT by 16 hours. Thus the action of these two cytokines on *SAACAT* was clearly synergistic (i.e. induction was greater than the sum of the individual inductions). The expected mean additive effect over these experiments for IL1 and IL6 was 6.2 fold for *SAA1CAT*440 , hence the degree of synergy calculated as the ratio of the observed induction to that expected if the effects were additive was approximately 3. Within individual experiments the degree of synergy was found to be in the range from 2 to 8.

Treatment of transfected cells with $100Uml^{-1}$ of rhTNFα gave a 2 to 4 fold increase over constitutive levels for *SAA1CAT*440. Addition of $1Uml^{-1}$ at the same time induced CAT levels to 1 to 2 fold above the expected additive response. When IL6 and TNF were added concomitantly at the same concentrations as previously a 1 to 5 fold greater than additive response was obtained. Addition of all three cytokines stimulated to the same level as IL1 and IL6 together (figure 5.5).

The effect of IL1 and IL6 on HepG2 cells transiently transfected with *SAA1CAT*440 over a time course of 24 hours was studied, the results are presented in figure 5.6. Both cytokines induced maximal CAT activity approximately 15 to 20 hours after addition, with some residual expression being detectable at 24 hours. When the two cytokines were added together the pattern of expression was comparable but the

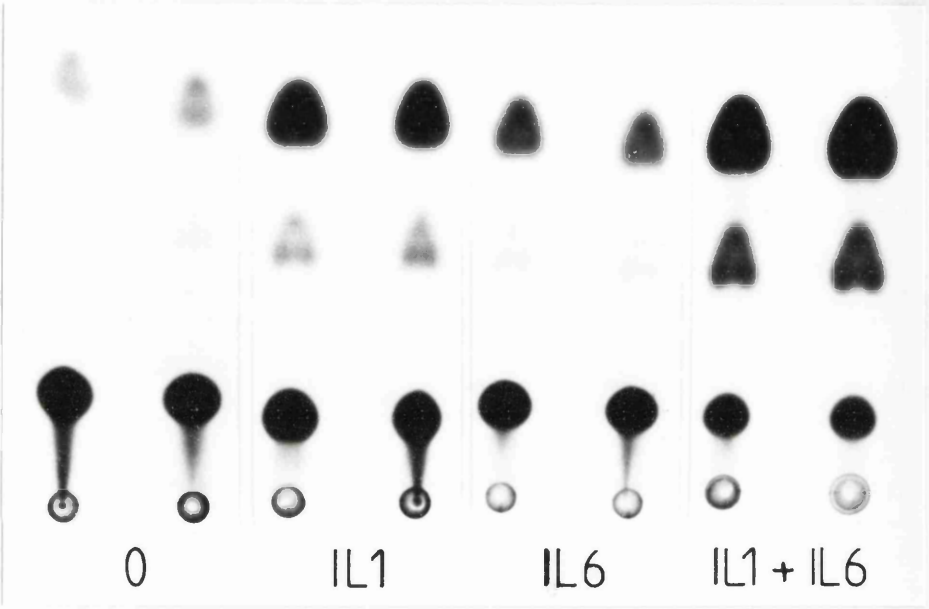


Figure 5.3 Induction of a CAT Reporter Gene Directed by the Human *SAA1* Promoter.
Cellular extracts from HepG2 cells transfected with *SAA1CAT*440 were assayed for CAT activity using [^{14}C]chloramphenicol as substrate in the presence of acteyl CoA. Reaction products were separated by thin layer chromotography and the acetylated (upper two spots) and unreacted chloramphenicol (lower spot) visualized by autoradiography. Products from wells transfected in duplicate and treated as indicated are shown.

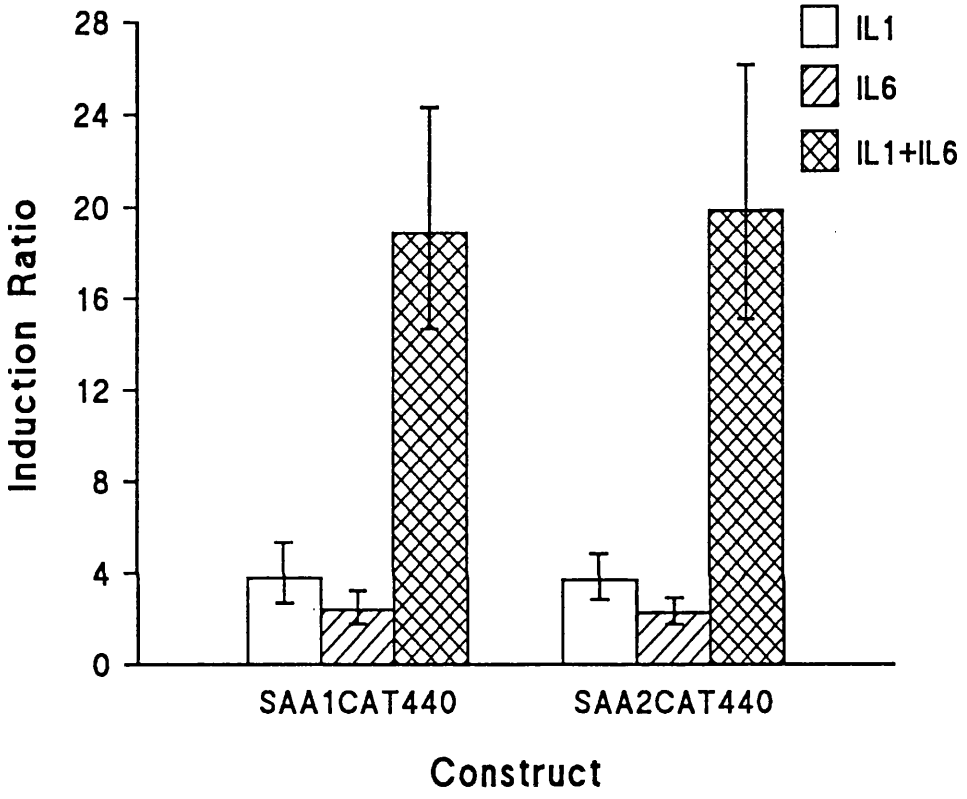


Figure 5.4 Effects of IL1 and IL6 on *SAACAT* constructs.
SAA1- and *SAA2CAT440* were transiently transfected into HepG2 cells and treated for 16h with IL1ß ($1Uml^{-1}$) and IL6 ($100Uml^{-1}$). The mean ratio for $n \geq 8$ experiments is shown with error bars spanning the 95% confidence interval.

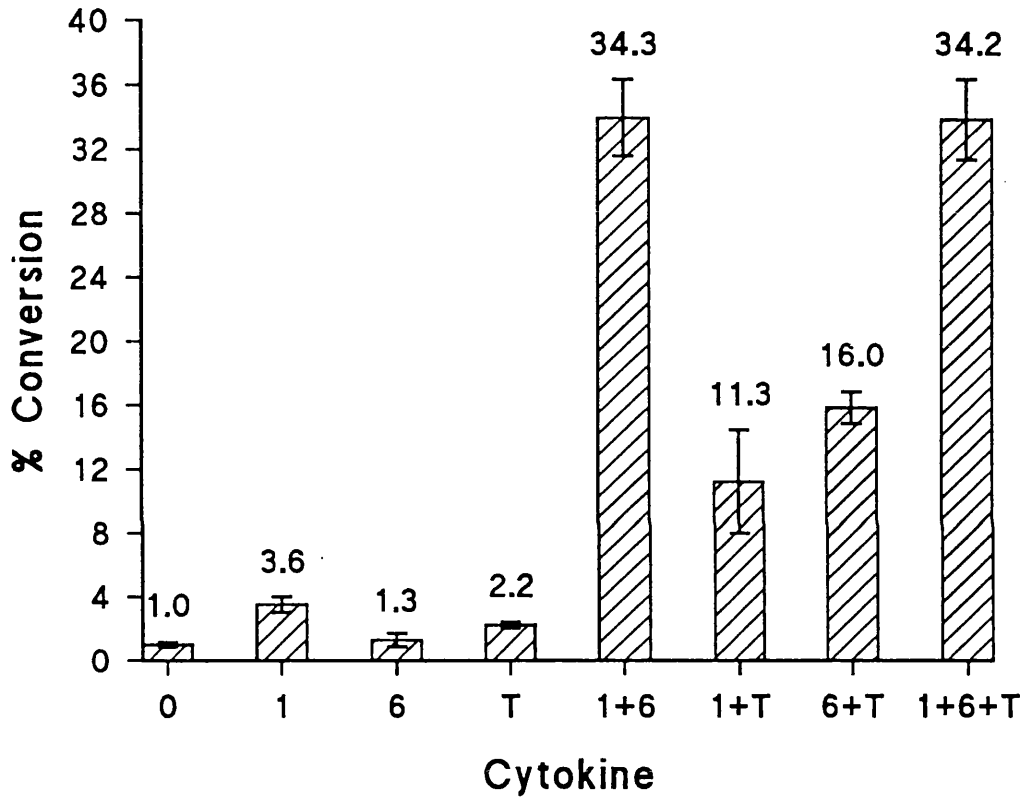


Figure 5.5 Response of *SAA1CAT440* to IL1ß, IL6 and TNFα.
HepG2 cells transiently transfected with *SAA1CAT*440 were treated with combinations of IL1ß (1), IL6 (6), TNFα (T) or were untreated (0). Bars represent the mean conversion of chloramphenicol to acetylated forms with error bars showing σ^{n-1}. The induction ratio is shown above each bar.

magnitude of the response was greatly amplified. An increase in activity was easily detectable 4 hours after stimulation and peaked at approximately 30 fold above constitutive levels at 15 hours, with a >20 fold stimulation being sustained to 24 hours. This transcriptional response seen in the transfected HepG2 cells thus preempts the peak in SAA serum levels measured following an inflammatory stimulus which peaks after 1 day (McAdam *et al*, 1978).

To gain further information with respect to the cytokine response of the *SAA1* gene the reporter gene constructs *SAA1CAT*1600, 800 and 160 were also transfected into HepG2 cells and stimulated with IL1 and IL6 for 16 hours. The results shown in figure 5.7 demonstrate that they are all inducible by IL1 and IL6 within a similar range of induction as *SAA1CAT*440. In addition the synergistic response to these two cytokines was maintained for *SAA1CAT*1600 and 800. However the evidence for a synergistic response with *SAA1CAT*160 was equivocal. Of four individual experiments carried out with this construct the degree of synergy measured was 3.4 and 5.5 in two experiments however in the other two experiments the induction was less than additive (whereas other constructs within the same experiment were synergistically induced). The reasons for these observations with *SAA1CAT*160 were unclear, the structural integrity of this construct was demonstrated by DNA sequencing and in each experiment there was a clear stimulation by IL1 and IL6 alone. The construct may partially disrupt a *cis*-acting sequence interfering with local secondary structure which only allowed factor binding on some occasions. However this does not really account for the "all or none" effect observed. Interestingly the 5' limit of this construct occurs at the SmaI site in a region which was found to be important in IL6 induction (*vide infra*).

The results obtained therefore demonstrated three effects:
i) that *SAA1* and *SAA2* responded in a similar fashion to three cytokines, IL1, IL6 and TNF (TNF induction of *SAA2* has been demonstrated by other members of the laboratory) all these cytokines are involved in the inflammatory response;
ii) combinations of cytokines were synergistic in induction of *SAACAT*, this could provide a molecular basis for the large increases in *SAA* seen during the APR. In the experiments carried out the synergy obtained with IL1 and IL6 was greater than either of these cytokines with TNF, however dose response experiments were not carried out for the TNF induction and thus a higher level of synergy may occur at different concentrations. A dose response experiment carried out with varying levels of IL1 and IL6 showed that the degree of synergy was greater at lower concentrations;
iii) that all the information required for induction by IL1 and IL6 is contained within 440bp upstream of exon 1.

5.2.2.3 Delineation of Elements Required for IL6 Induction of SAA.

Previous work in this laboratory has shown that IL1 modulation of SAA transcription occurs via the NFκB binding site, however the site of IL6 action was unknown. Therefore to address the question of how IL1/IL6 synergism occurred at the

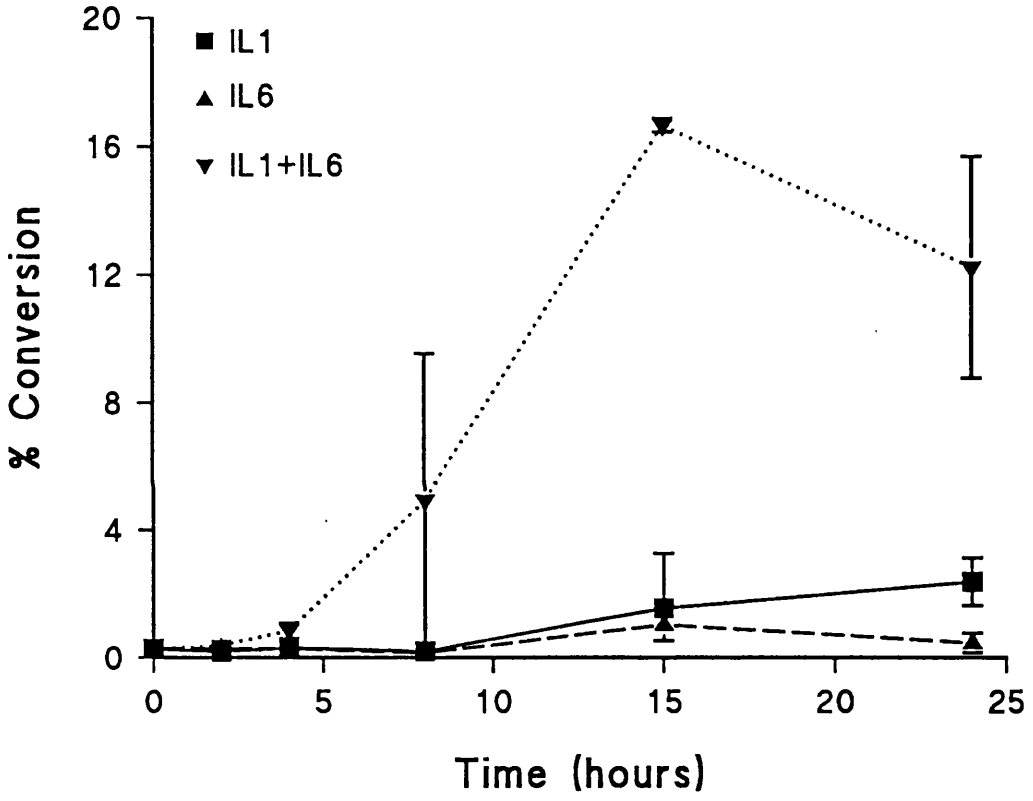


Figure 5.6 Time course of *SAACAT* induction by IL1 and IL6.
A representative time course experiment is shown for the activity of *SAA1CAT440* transfected HepG2 cells following addition of IL1ß/IL6 at time zero with harvesting of cells over a 24h period. Error bars represent σ^{n-1}.

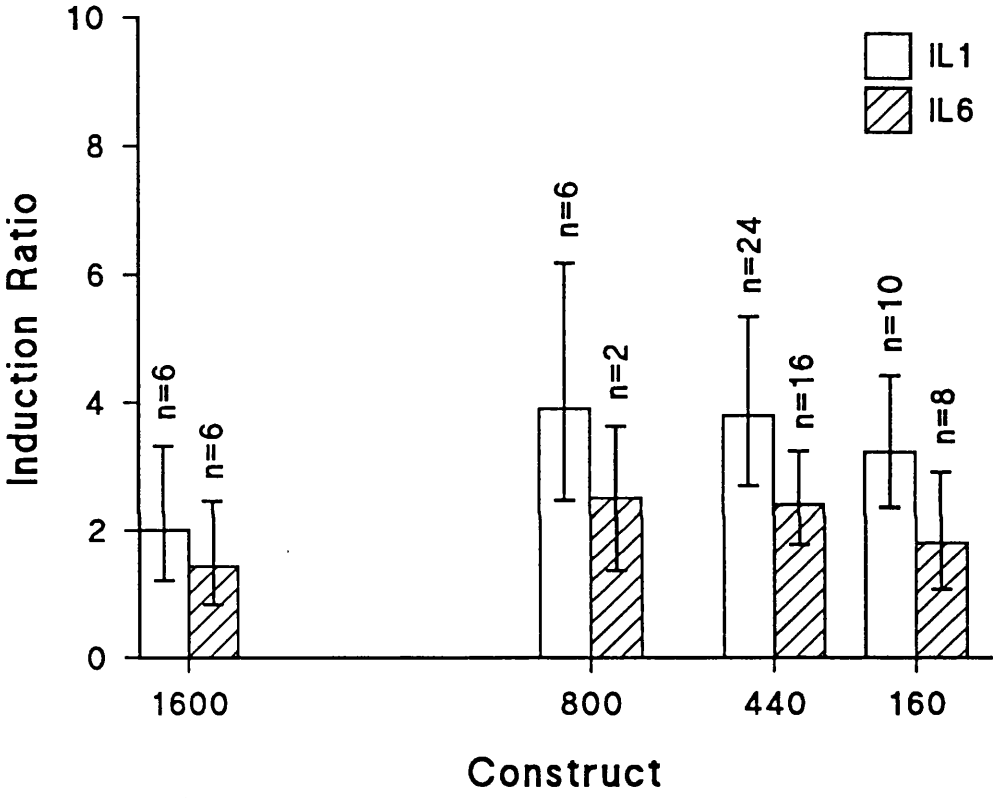


Figure 5.7 Induction of *SAA1CAT* deletion constructs by IL1 and IL6. *SAA1CAT* constructs containing various lengths of *SAA1* 5'-flanking region as shown on the abscissa were transiently transfected into HepG2 cells and treated with IL1ß/IL6 for 16h. The mean induction ratios are shown with 95% confidence interval for n experiments.

level of transcription it was decided that IL6 effects should first be defined. When this work commenced the sequence element CTGGGA was the centre of attention in a number of laboratories which were attempting to elucidate potential *cis*-acting sequences involved in IL6 induction of acute phase genes. This element was present in the *SAA* promoter (see figures 3.5 and 5.1) and thus regarded as a potential target for IL6 induced pathways.

Using the transient transfection system and the *SAA2CAT* constructs the level of IL6 inducibility was compared for a construct containing the putative IL6RE (*SAA2CAT*440) with the construct *SAA2CAT*100 which lacks this region. Transfected cells were stimulated with IL6 for various times over a 32 hour period and assayed for CAT activity. The results for 3 individual experiments are presented in figure 5.8. *SAA2CAT*440 was inducible by IL6 with maximal expression occurring 20-24 hours after stimulation. In contrast *SAA2CAT*100 showed little stimulation above constitutve levels until 24 hours when 50% of the *SAA2CAT*440 level was achieved. Indeed at 16 hours after stimulation activity was consistently below the uninduced level. These results indicated that there were *cis*-acting sequences present between -100 and -440bp in the *SAA2* promoter which were important for IL6 induction of transcription. The PstI site used to clone *SAA2CAT*100 falls at the 5' boundary of the NFκB site however the reduced IL6 effect could not be attributed to disruption of NFκB binding as this construct was fully inducible by IL1 where the integrity of this site is critical (see figure 5.10). Obviously the differential responses of the two constructs could have been attributable to sequences anywhere within the 340bp region missing from *SAA2CAT*100 and not involved the putative IL6RE *per se*. Further evidence for this region having a role in IL6 induction was obtained in nuclear factor binding studies (see below).

Potential involvement of the NFκB element in IL6 modulation of transcription was also investigated. The binding site for NFκB GGGACTTTCC had been mutated to CTCACTTTCC in the *SAA2* promoter by site-directed mutagenesis, this mutation had previously been demonstrated to block binding of NFκB (Nabel and Baltimore, 1987). The effect of IL6 on *SAA2CAT*440 (wild-type) and *SAA2CAT*440m (mutated NFκB site) in HepG2 cells is shown in figure 5.8. Like *SAA2CAT*100 the mutant showed a reduced level of inducibility over the whole time course compared to the wild-type construct although induction was measurable towards the latter time points. This contrasted with the IL1 effect which gave no induction with *SAA2CAT*440m (fig. 5.10). Thus the NFκB site was required to achieve the maximal IL6 effect but there was also a residual level of induction in the absence of function of this *cis*-acting sequence. Analysis of variance for these results showed that there was a highly significant difference between *SAA2CAT*440 and *SAA2CAT*440m and *SAA2CAT*100 ($p=0.002$) but *SAA2CAT*440m and *SAA2CAT*100 were not significantly different from each other ($p=0.133$). In addition the linear trend over time was not significantly different between constructs.

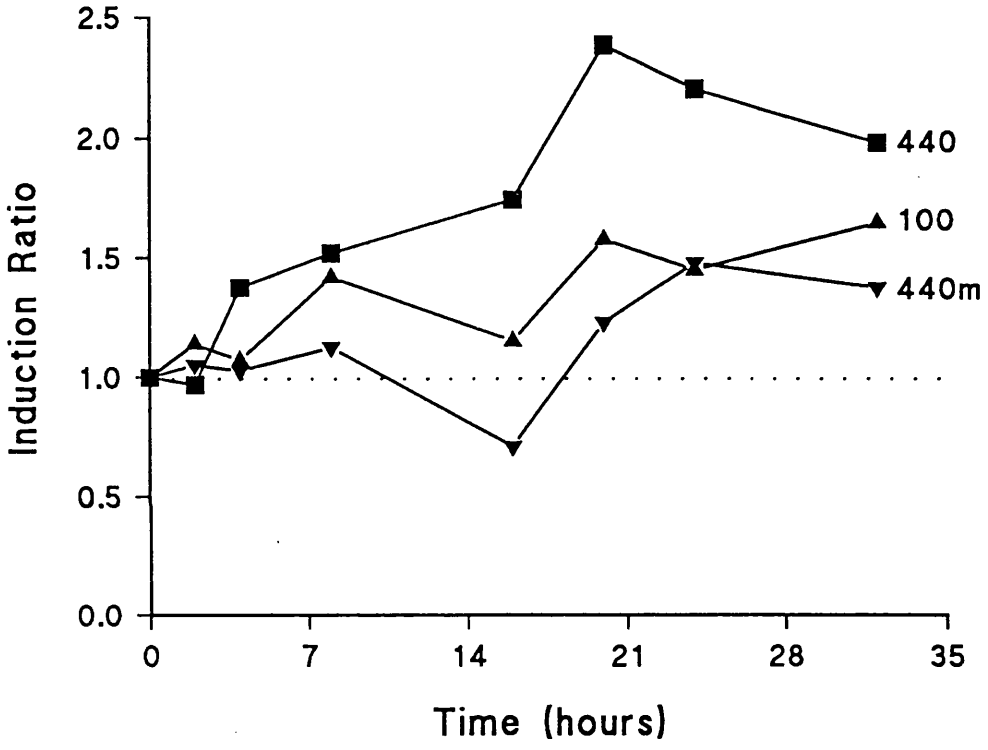


Figure 5.8 Time course of induction of *SAA2CAT* constructs by IL6.
The constructs *SAA2CAT*440, 440m and 100 were transiently transfected into HepG2 cells and CAT activity measured after various incubation times with $100Uml^{-1}$ IL6. The values shown were calculated from the means of 3 individual experiments carried out on duplicate wells.

The data obtained from these experiments clearly demonstrated the involvement of two regions in the IL6 modulation of SAA transcription. Firstly sequences between -100 and -440bp which contained the putative IL6 responsive element (IL6RE) and secondly the NFκB binding site. In both cases deletion of the sequences reduced inducibility, further studies were required to determine whether inducible factors bound at these sequences (see below).

5.2.2.4 Involvement of the IL6RE and the NFκB Binding Site in IL1/IL6 Synergism.

The synergistic activation of *SAACAT* could be a result of interaction between the pathways of the two cytokines at one or more potential levels:

i) increased receptor recruitment, for example, IL1 could upregulate the IL6R in HepG2 cells or *vice versa*. Such mechanisms have been demonstrated in other systems such as the upregulation of TNFR by IFNγ in synergism between these two cytokines (Esparza *et al*, 1987);

ii) between factors in the second messenger pathways. Although these remain obscure for IL1 and IL6 there maybe crossover between the two pathways leading to priming effects. For example TNFα can upregulate a possible component of some PKC signalling pathways (myristoylated alanine-rich C kinase substrate, MARCKS) thus priming cells for subsequent activation by PKC activators (Thelen *et al*, 1990);

iii) cooperativity between transcription factors induced by IL1 and IL6 with the transcriptional machinery. Many examples are known where the presence of multiple TF binding sites in a promoter gives a response which is much greater than the additive effects of the individual sites acting alone (for example, see Carey *et al*, 1990).

iv) other post-transcriptional mechanisms. For example one of the cytokines could increase transcription and the other increase the stability of the mRNA thus leading to large increases in response. mRNA stabilizing effects have been suggested for SAA during the APR in mice (Lowell *et al*, 1986b; Rienhoff and Groudine, 1988). In addition IL1ß regulates mRNA decay of cytokines such as *gro* (Stoeckle, 1991).

Although interaction may occur at the level of receptor recruitment or second messengers, these pathways ultimately converge at the SAA promoter where the increase in transcription is mediated. In addition it could be argued that although effects on mRNA half-life or translational mechanisms may occur *in vivo* such events occurring in this system would have to be general/non-specific effects as the gene product was CAT. The latter proposal was tested by transfecting TK-CAT, a construct containing the thymidine kinase promoter but lacking any SAA sequences, into HepG2 cells and stimulating with IL1 and IL6. The results of these experiments are shown in figure 5.9, *SAA2CAT*440 was used as a positive control. TK-*CAT* gave a high level of CAT activity, however this was not increased by treating cells with IL1, a small increase was seen with IL6 alone but no large increase was seen when both cytokines were added. This demonstrated that the synergistic effect was specific to the SAA promoter, although exon 1 of SAA was present in the reporter genes and thus may potentially have an effect when present in the mRNA.

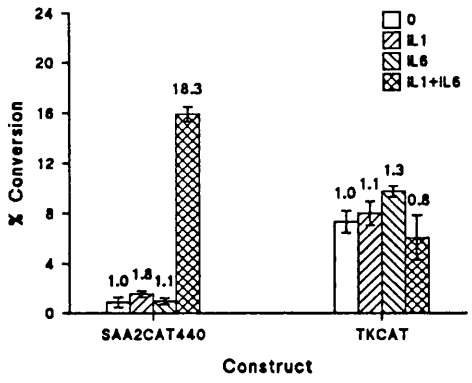


Figure 5.9 Specificity of the *SAACAT* response to IL1 and IL6.
a) CAT activity in HepG2 cells transfected with *SAA2CAT*440 or *TKCAT* and treated with the cytokines shown for 16h. Bars show mean $\pm \sigma^{n-1}$ with induction ratios shown above each bar.

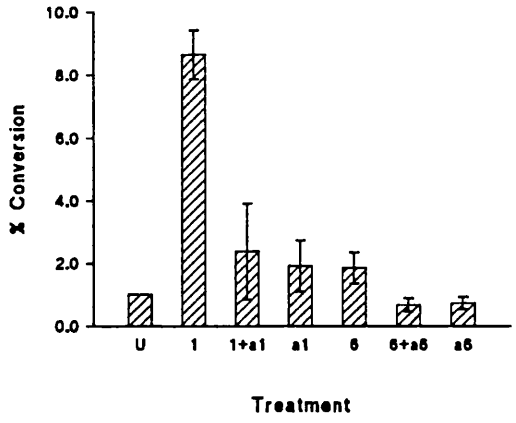


Fig. 5.9 (cont.) b) The response of *SAA2CAT*440 transfected HepG2 cells to IL1 and IL6 could be inhibited by the addition of specific antisera. Bars show mean $\pm \sigma^{n-1}$. 1=IL1, a1=anti-IL1ß antibody, 6+IL6, a6=anti-IL6 antibody.

The NFκB site was crucial for IL1 induction and also required for maximal IL6 induction. In order to confirm a requiremnt for the NFκB site in the synergistic effect *SAA2CAT*440m was transfected and stimulated. The results shown in figure 5.10 clearly demonstrate that the NFκB site is an absolute requirement. The IL1 stimulation was abolished, the IL6 effect reduced and the addition of both cytokines together did not markedly increase the induction. Because IL1 effects require NFκB binding, this result was not unexpected and thus showed that IL1 was not inducing other factors which interacted to produce the synergy, if other factors were induced then their action was dependent on the NFκB site.

The functional studies using *SAA2CAT*100 described above indicated that sequences upstream of -100 were involved in mediating IL6 activity. Thus synergistic activation may also require *cis*-acting sequences in this region. Cells transfected with *SAA2CAT*100 gave synergistic induction with IL1 and IL6 in the same range as *SAA2CAT*440 *i.e.* up to 40 fold (figure 5.10). However IL1 stimulation of *SAA2CAT*100 was much higher (mean of 10.97 compared to mean of 3.72 for *SAA2CAT*440). Hence when the degree of synergy was calculated (ratio of measured effect of IL1 + IL6 to expected additive effect) a value of 2.2 is obtained for *SAA2CAT*100 compared to 3.3 for *SAA2CAT*440. This indicates the possible involvement of sequences upstream of -100 in the mechanism of synergism.

The IL1 induction was much greater for *SAA2CAT*100 than *SAACAT*440, this may be due to *cis*-acting sequences which mediate a repressor function within this 340bp region. There was no evidence for an increased basal level of transcription with this construct. It is not known whether the degree of synergism would have been lower in the absence of the increased IL1 effect. A generally recognized phenomenon is that synergy reduces as the quantitative effects of the individual components increases, possibly due to saturation effects. Despite the fact that a vast number of promoters were transfected into the cells only a small number are transcribed, hence saturation may be occurring at the level of transcription. Consequently an increase in the IL1 effect would give no measurable increase in overall synergy. Thus the involvement of upstream sequences is not definitive on the basis of the current data. Mutagenesis of the putative IL6RE in *SAA2CA*T440 will be necessary to demonstrate this.

These results identified the NFκB site as an essential promoter element in mediating the synergistic effect. Binding at the NFκB site induced by IL1 may be enhanced by IL6, alternatively IL6 may induce distinct factors which act cooperatively with the transcriptional machinery. In order to gain further information on these possibilities nuclear factor binding studies were carried out.

5.2.2.5 Priming of Cytokine Signalling Pathways by IL1 and IL6.

The signalling pathways activated by IL1 and IL6 in HepG2 cells converge at the SAA promoter to activate transcription, however "cross-talk" may also occur at points more proximal to the receptors. The synergistic effect of IL1 and IL6 maybe mediated in

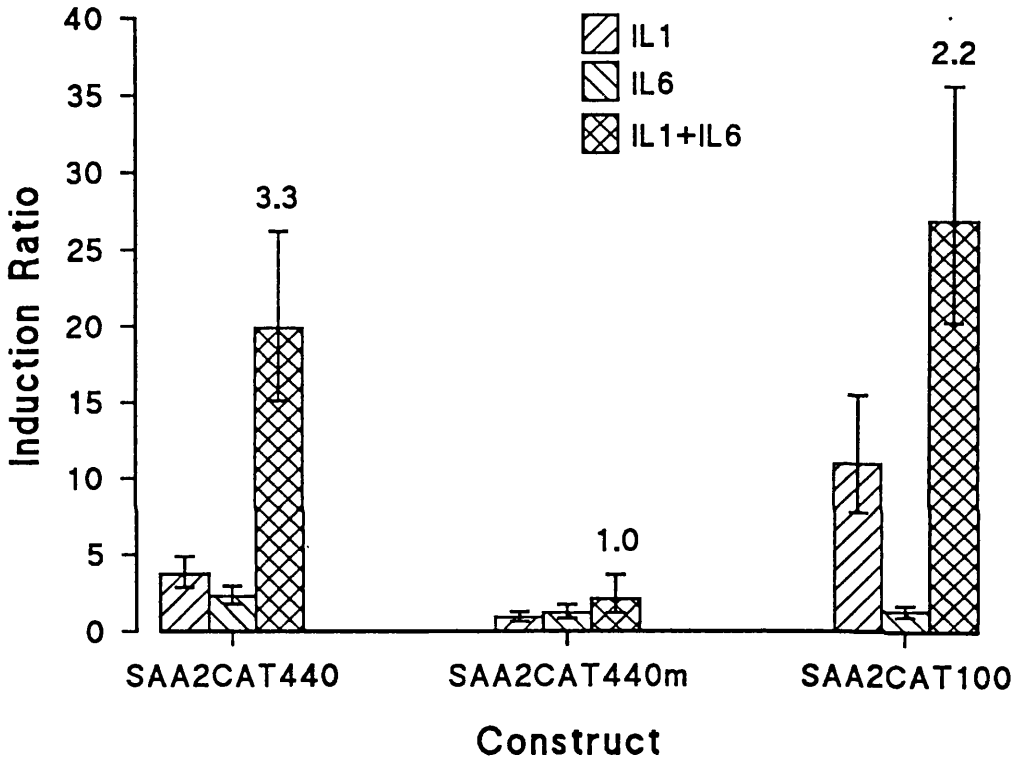


Fig. 5.10 Involvement of the NFκB motif and upstream sequences in the synergistic induction of SAA by IL1 and IL6.
CAT induction ratio of HepG2 cells transfected with *SAA2CAT*440, 440m and 100 and treated with cytokines as indicated for 16h shown as mean ± 95%c.i. Values above the IL1+IL6 bars show the degree of synergy calculated using the mean values represented.

part by "priming" by one cytokine of the signalling pathway of the other. For example IL1 may make factors available for the subsequent rapid utilization by the IL6 pathway. Preliminary investigations were carried out to shed some light on this question by carrying out "delayed addition" experiments. HepG2 cells transfected with *SAA1CAT*440 were treated with IL1 and IL6 for various times between 14 hours and 2 hours. Other wells were treated with IL1 for 14 hours and IL6 added at various time points after as shown in figure 5.11. For example in the 11 hour delayed addition experiment IL1 was added to the wells and IL6 added 3 hours later in the continued presence of IL1 with the cells being harvested after a further 11 hours. Induction was also measured for treatments with IL1 and IL6 alone. The results showed that 3 or 6 hour treatment with IL1 followed by 11 and 8 hours treatment with IL6 (respectively) significantly enhanced the induction level achieved by addition of the two cytokines together for 11 or 8 hours. This was not true for 10 or 12 hours IL1 pretreatment followed by the addition of IL6 for 4 and 2 hours respectively, in these cases although response was enhanced above that for the addition of IL1 and IL6 together for 4 and 2 hours, this could be attributed to the additive presence of IL1 for 14 hours plus IL6 for 4 or 2 hours. Experiments where IL6 was added between 13 hours and 55 minutes and 12 hours prior to harvesting with IL1 being present for 14 hours gave essentially the same level of induction as both cytokines being together for 14 hours (data not shown).

Similar delayed addition experiments adding IL1 at various time points after the addition of IL6 which was present for a total of 14 hours gave comparable results with the absolute level of induction being enhanced over that when the cytokines were present for the same time. This effect was most marked when addition of IL1 was delayed for 3 hours after the addition of IL6.

The degree of synergy was not significantly different at each time point for wells where the cytokines were present for the same time period compared to the delayed addition samples. However the results demonstrated that the cytokines could still mediate a synergistic effect even when there was a 6 hour delay between addition. This effect was lost if the lapse time was extended to 10 hours. However interaction of the two cytokine pathways at lapse times greater than 10 hours cannot be definitely ruled out: in the experiments carried out there may not have been a sufficient incubation time between addition of the second cytokine and harvesting of the cells to measure the full induction effect. Nevertheless IL1 effects were still evident for some time and IL6 mediated events could interact with these. This may have represented interaction of transcription factors, second messengers or both. The first cytokine added was not removed before the addition of the second thus the results may represent continued stimulation by the first cytokine in conjunction with the second, alternatively priming of signalling pathways maybe occurring.

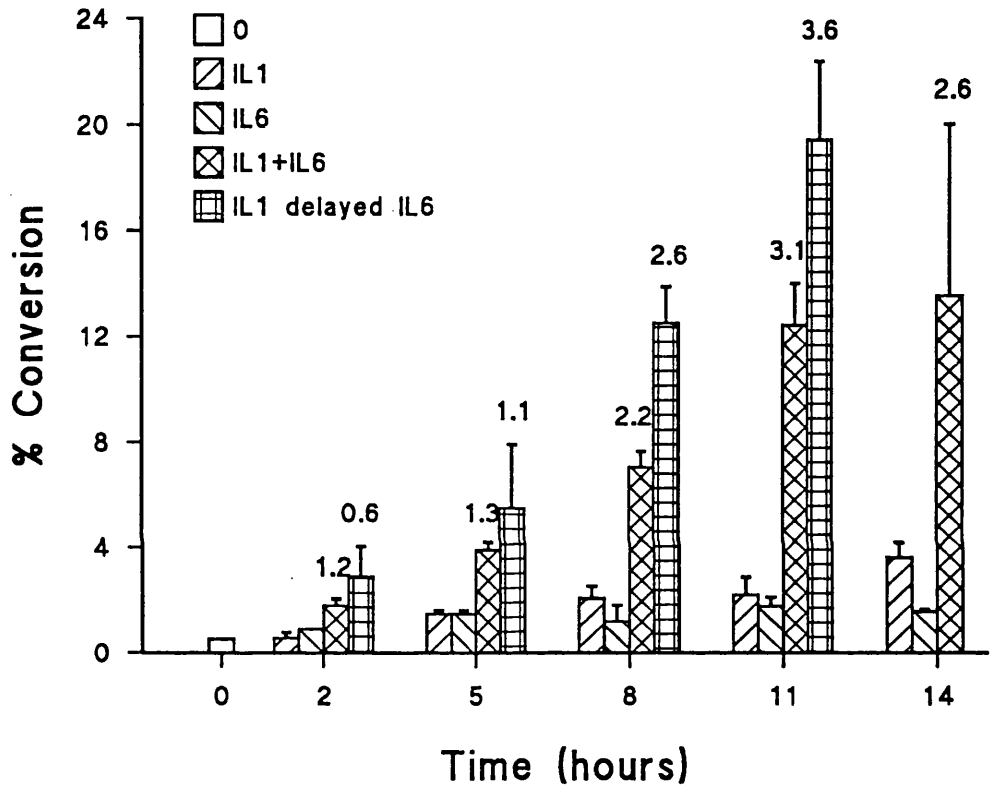


Figure 5.11 IL1 and IL6 delayed addition experiment.
HepG2 cells transiently transfected with *SAA1CAT*440 were treated with IL1/IL6 for the times shown. In delayed addition wells IL1 was present for a total of 14h with IL6 being present for the time shown. Thus for the IL1 delayed IL6 column at 11h, IL6 was added 3h after IL1 and incubation continued for a further 11h prior to assay. For all other columns at the same time point all cytokines were present for 11 hours. Each bar shows the mean $\pm \sigma^{n-1}$. Values above the bars represent the degree of synergy calculated using the mean values of the IL1 and IL6 results for IL1+IL6 bars or mean values of IL1 at 14h with that for IL6 at each time point for IL1 delayed IL6 bars.

5.2.3 Studies on Nuclear Factor Binding to the SAA 5' Region.

5.2.3.1 Proteins in IL6 Treated HepG2 Cell Nuclear Extracts Bind the *SAA2* Promoter in a Novel Manner.

IL1 induced HepG2 cell nuclear extracts show a single inducible factor binding to a 265bp fragment extending from -236 to +29 of *SAA2*. DNA footprinting and mutational analysis showed that this binding was to the NFκB binding site (Edbrooke *et al*, 1991). In order to investigate the binding characteristics of nuclear factors from IL6 induced cells, HepG2 cells were induced with 100Uml^{-1} of IL6 for various times between 0 and 24 hours and nuclear proteins prepared. Electrophoretic mobility shift assays (bandshifts) were carried out using the 265bp *SAA2* promoter fragment (containing the NFκB site, putative IL6RE, TATA box etc.) end-labelled with ^{32}P incubated with nuclear proteins prepared between 5 minutes and 24 hours after stimulating with IL6 (figure 5.12). Two shifted bands were observed which were faint at 5 minutes increased slightly at 30 minutes with a definite quantitative increase in binding visible at 8 hours which was maintained through 16 hours but had returned to the constitutive level by 24 hours. A PMA stimulated HeLa cell nuclear extract was used as a positive control for NFκB binding.

The pattern of binding obtained with the IL6 extracts had several characteristics which were distinct from the binding seen following IL1 treatment. Firstly, two shifted bands were evident whereas only one was seen with the IL1 extracts. Secondly the time course of binding differed in that IL1 induces rapid binding which is detectable after $\leq$15 minutes stimulation but is essentially absent by 2 hours, in contrast maximal binding with the IL6 extracts was observed between 8 and 16 hours. However the lower band had a similar mobility to the IL1 induced NFκB-like factor and PMA induced NFκB binding as seen on bandshift gels. In addition the functional assays indicated a role for the NFκB site in mediating the IL6 response as mutation of it reduced CAT activity.

5.2.3.2 Binding of Nuclear Proteins to the NFκB Site Region.

To assess whether the NFκB site was involved in nuclear protein binding in the IL6 extracts the 265bp fragment carrying a mutated NFκB site (CTCACTTTCC) was used as a probe in bandshift experiments. Figure 5.12 shows that binding at all time points in the IL6 extracts was maintained but was abolished in the HeLa/PMA control. That is, nuclear protein binding in these extracts did not require the cognate NFκB binding site.

Competition experiments were also carried out using ^{32}P-labelled 265bp fragment containing the wild-type NFκB site as probe with 50 or 250ng of unlabelled double stranded SAAκB oligonucleotide as competitor (see section 2.2.2.3.2). Surprisingly 50ng of SAAκB oligonucleotide partially competed binding in both the extracts used (30 minute and 16 hour) whereas 250ng SAAκB competitor completely inhibited binding (figure 5.13). This was somewhat unexpected considering that proteins in these extracts bound the 265bp fragment containing a mutated NFκB site. Thus factors in the IL6

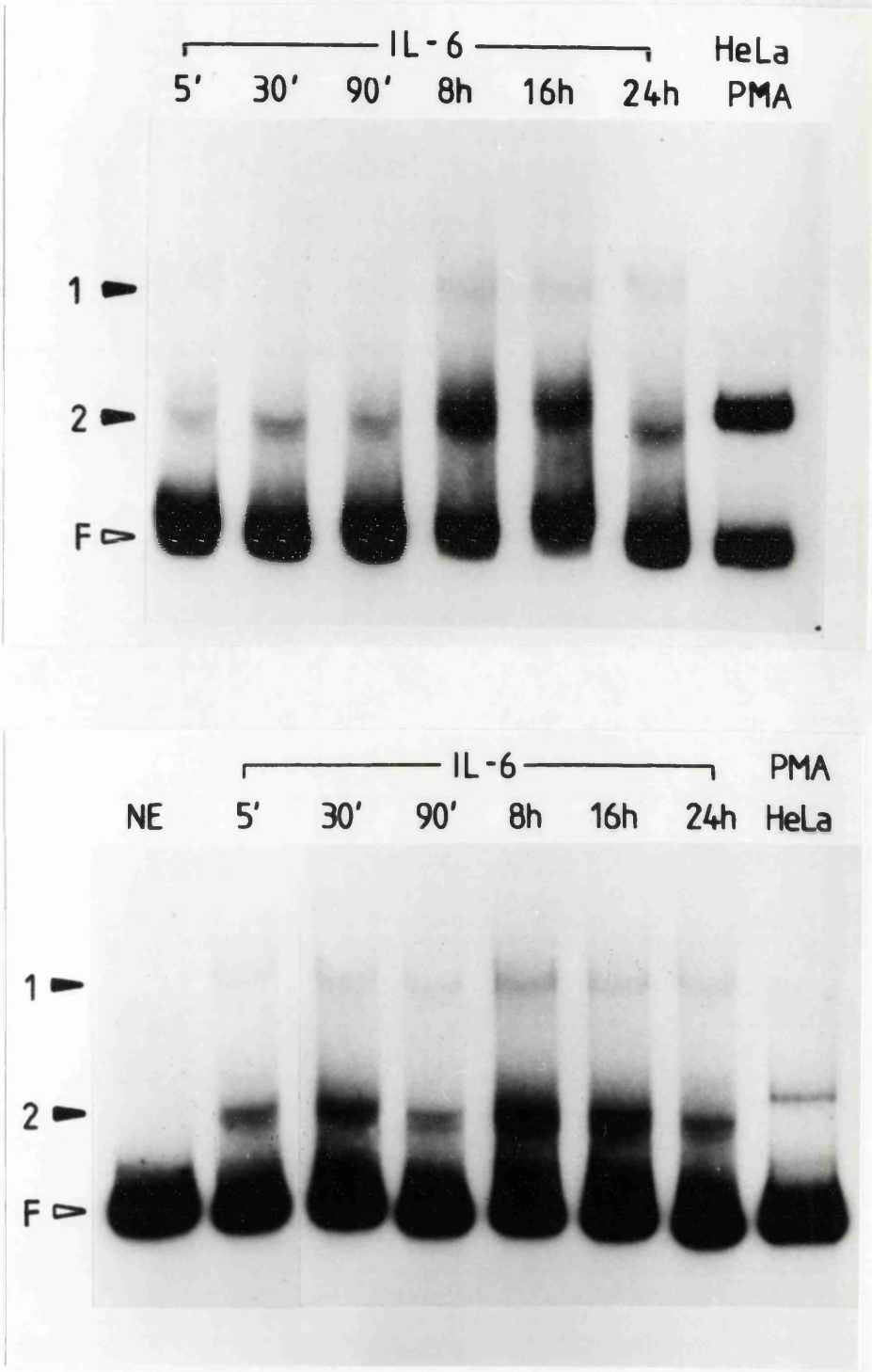


Figure 5.12 Bandshift Analysis of IL6 Treated HepG2 Nuclear Extracts.
Nuclear proteins from HepG2 cells treated with IL6 for the indicated times were used in bandshift assays with labelled 265bp fragment carrying wild-type (upper panel) or mutated (lower panel) NFκB sites. A PMA treated HeLa extract was used as a positive control for NFκB binding activity. 1 and 2 = shifted bands, F = free probe.

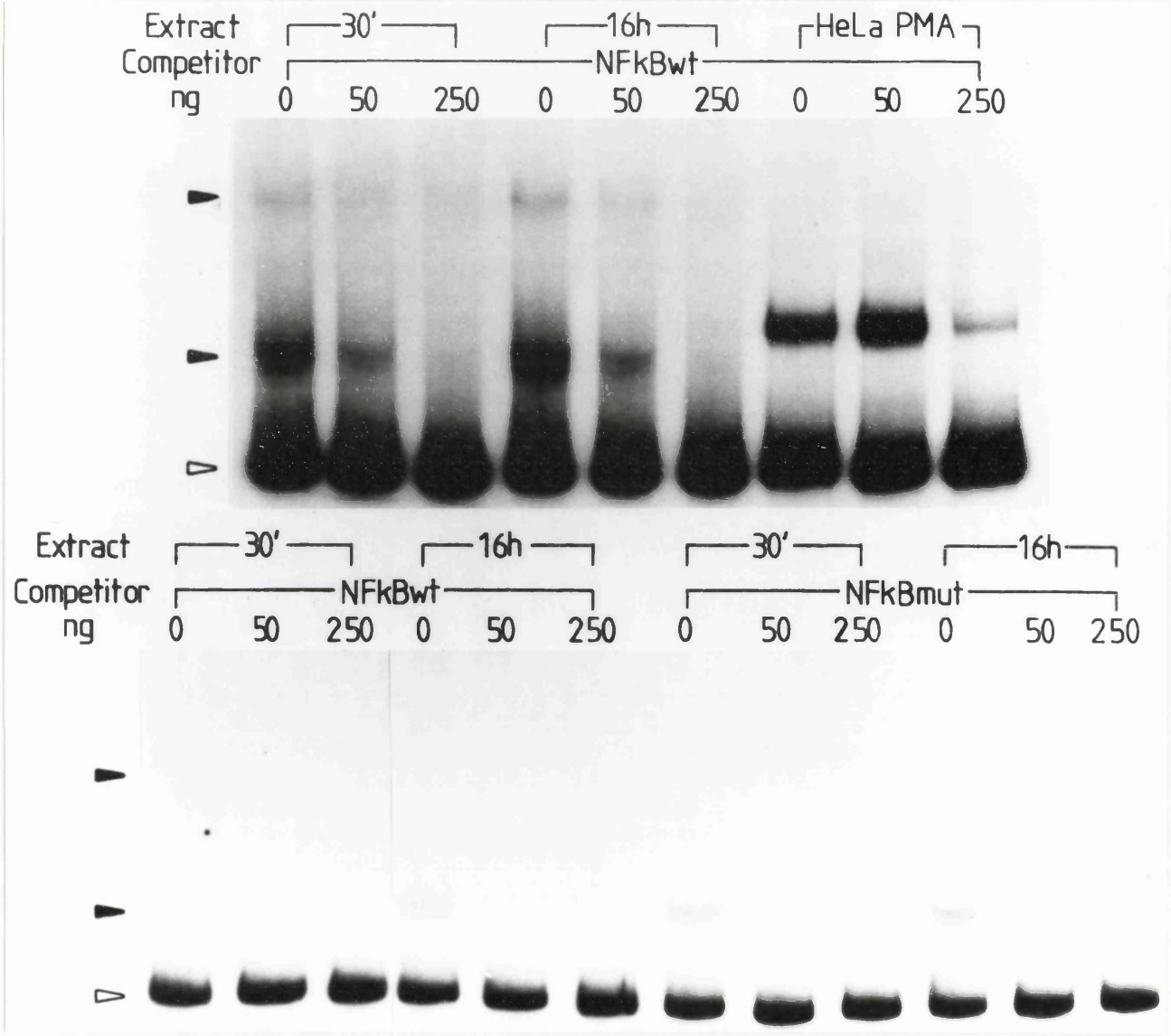


Figure 5.13 Specificity of Nuclear Factor Binding to the SAA 265bp Fragment: Competition by Unlabelled Fragments.
Bandshift reactions were carried out using HepG2 extracts treated with IL6 for 30 min and 16 hours and HeLa/PMA control (see figure 5.13). Reactions were preincubated with 0, 50 or 250ng of unlabelled NFκB oligonucleotide before the addition of labelled 265bp fragment. In the lower panel the experiment was repeated including the NFκB mutant oligonucleotide as competitior. Competition of the shifted bands (closed arrow heads) can be seen. Open arrow = free probe.

extracts could bind to sequences in the SAAκB 28mer which contained the sequence 5'-CTGCAGGGACTTTCCCCAGG-3' as a duplex (the remaining 8 nucleotides comprising BamHI 3' overhangs). When the sequence 5'-CTGCACTCACTTTCCCCAGG-3' is used as a cold competitor the IL1/HepG2 or HeLa/PMA nuclear extract protein binding is not competed. However the mutated NFκB site competed strongly for factors present in the IL6 30 minute and 16 hour extracts (figure 5.13). These observations therefore demonstrated that the binding was to the NFκB region but that the three guanine residues which are essential for binding of NFκB *per se* were not essential for the IL6 extract proteins. Thus the proteins were distinct from the IL1 induced nuclear factors.

To assess the binding to the NFκB oligonucleotide sequences further, these were end-labelled and used as probes in the band shift assays. A typical gel is shown in figure 5.14, binding to the wild-type SAAκB sequence, shwed differing mobility of the shifted bands compared with IL1 induced extracts. In addition binding to the mutated SAAκB sequence contrasts with the lack of binding in the IL1 extracts.

The 265bp fragment contained the NFκB site as well as the putative IL6RE. To determine whether binding occurred to the 5' half of the 265bp fragment it was cut at the PstI site and labelled. This provided a probe containing the putative IL6RE but lacking the NFκB site (i.e. -236 to -100 of *SAA2*). When this 126bp fragment was incubated with the IL6 extracts the pattern of binding was distinct from that obtained with the whole promoter region (figure 5.15). A strong band of low molecular weight was seen but an upper band was not identifiable. Also of note was the lack of variation in binding intensity over the time course. No binding was seen in the IL1 extracts to this fragment.

Thus in summary nuclear factors from IL6 induced cells contain proteins which bind at both the NFκB region and within a 136bp spanning from -236 to -100 which lacked the NFκB site. Binding to the complete 265bp SAA2 promoter fragment was increased at 8 hours after stimulation whereas binding to the 136bp fragment did not alter with induction. Binding at the NFκB site was distinct from NFκB binding in that it did not require the integrity of the three G residues. When the 265bp fragment carrying a mutated NFκB site was used binding intensity appeared to be increased in the IL6 extracts over that obtained with the wild-type fragment. It seems probable that the non-NFκB factors binding at the NFκB site would sterically compete for binding with NFκB itself, removal of any NFκB binding that was occurring by mutating the three G's would abrogate this competition and thus allow increased binding of the secondary factors(s).

To confirm these findings nuclear extracts were prepared from IL6 stimulated HepG2 cells and fur ther time points included to gain more information. These extracts showed a slightly different pattern of binding compared to those already analyzed. The difference was manifest in that the increase in binding at 8 and 16 hours was not as marked as previously, in addition the upper shifted band was of greater intensity in comparison to the lower band than previously. The IL6 used was from a different source (British Biotechnology) and the unit activity was weaker as determined using the

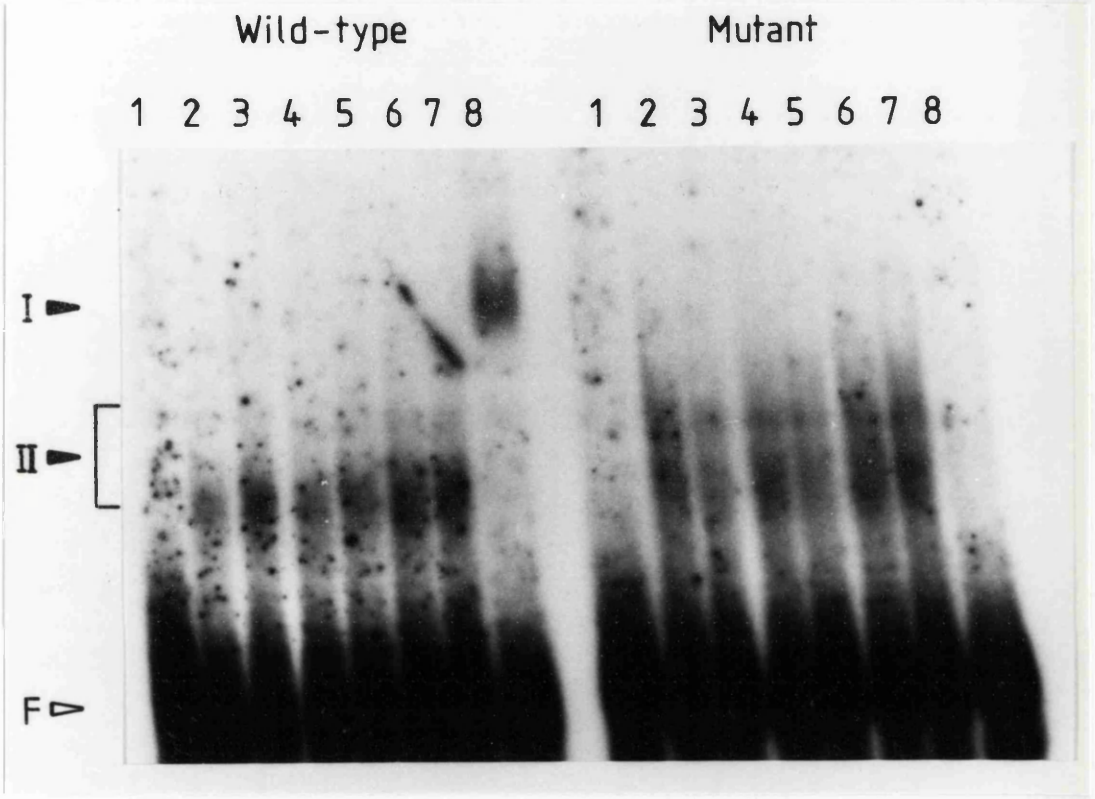


Figure 5.14 IL6 Treated HepG2 Cell Nuclear Proteins Bind to Wild Type and Mutant NFκB Site Oligonucleotides.
Band shift reactions were carried out in the presence of labelled double stranded oligonucleotides to the wild-type and mutated NFκB sites (see section section 2.1.5 for sequences). Lanes 1-7 = untreated, 5', 30', 90', 8h, 16h, 24h IL6/HepG2 extracts, 8 = IL1/HepG2 extract (positive control for NFκB activity). I and II = shifted complexes, F = free probe.

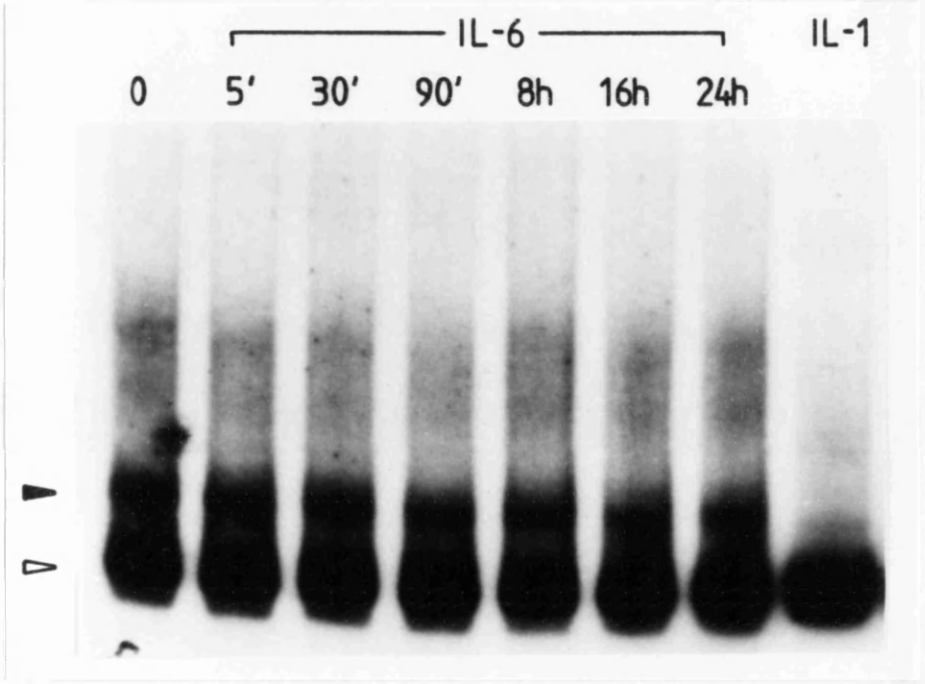


Figure 5.15 IL6 Treated HepG2 Cell Nuclear Proteins Bind to the 136bp PstI Fragment Lacking the NFκB Site.

A single shifted band (closed arrowhead) was observed when the IL6 extracts were incubated with labelled 136bp PstI fragment (-236 to -100 of the *SAA2* promoter) lacking the NFκB site

transient transfection system, this may explain the lack of a marked increase in binding at 8 hours. However the binding specificities to the various promoter fragments remained the same. There was a slight reduction in binding intensity in the 5 minute extract compared to constitutive demonstrable with the 265bp fragment but this had increased again by 4 hours after stimulation (figure 5.16A). Binding to the 265bp fragment containing the mutated NFκB site showed the same trends. Nuclear factors in these extracts were also found to bind the wild-type and mutant double stranded NFκB oligonucleotides (figure 5.16B), the binding was much clearer than that obtained with the previous extracts. For the wild-type NFκB site 2 shifted bands were visible, the upper band had the same mobility as the NFκB band in the IL1 extract. The lower (complex II) band was also present at a low level in the IL1 extract. With the mutated NFκB site the upper band was absent whereas the lower band remained. This result suggested that the lower mobility protein-DNA complex represents binding of an NFκB-like factor, with the faster migrating complex having distinct properties (*i.e.* does not require the three G residues of the NFκB site to be intact) in binding the NFκB region. There was also evidence from these gels that the upper band was inducible at the early time points with decreased binding by 16 and 24 hours. However this potential inducibilty was minimal compared to that obtained with IL1 treatment and further extracts would have to be made to confirm this.

5.2.3.3 Footprint Analysis of Nuclear Protein Binding to the *SAA2* Promoter.

Footprinting techniques provide a rapid method of defining nuclear protein binding sites to identify regions of importance. To this end methylation interference footprinting was carried out using ^{32}P end labelled 265bp *SAA2* promoter fragment. By carrying out a protein-DNA binding reaction with the probe partially chemically methylated at G residues (Maxam and Gilbert, 1980) the shifted products will contain only fragments with non-methylated Gs in the binding sequence (assuming the binding of protein to the DNA is methylation sensitive). The binding sequence is subsequently uncleavable by piperidine creating a gap in the sequence ladder. Methylation interference footprinting was carried out using the 8 hour IL6 nuclear extract but no clear footprinted region was seen. However there were problems with this method in that there were few Gs in the putative IL6RE region whose sequence could be obtained from a nearby site which could be end labelled. To overcome this problem an alternative method was employed whereby a second Maxam and Gilbert reaction was used to modify C and T residues with hydrazine. This cleaves the pyrimidine ring and eventually leads to the conversion of sugars in the DNA backbone to hydrazones (Maxam and Gilbert, 1980). Theoretically by analogy with methylation interference this would interfere with binding of any nuclear factors to C and T nucleotides. Thus labelled DNA was treated with hydrazine, incubated with nuclear proteins from the 8 hour IL6 extract and shifted bands eluted. Following strand separation and cleavage with piperidine the products were run on a sequencing gel. A region was clearly visible where intensity of C and T residues was

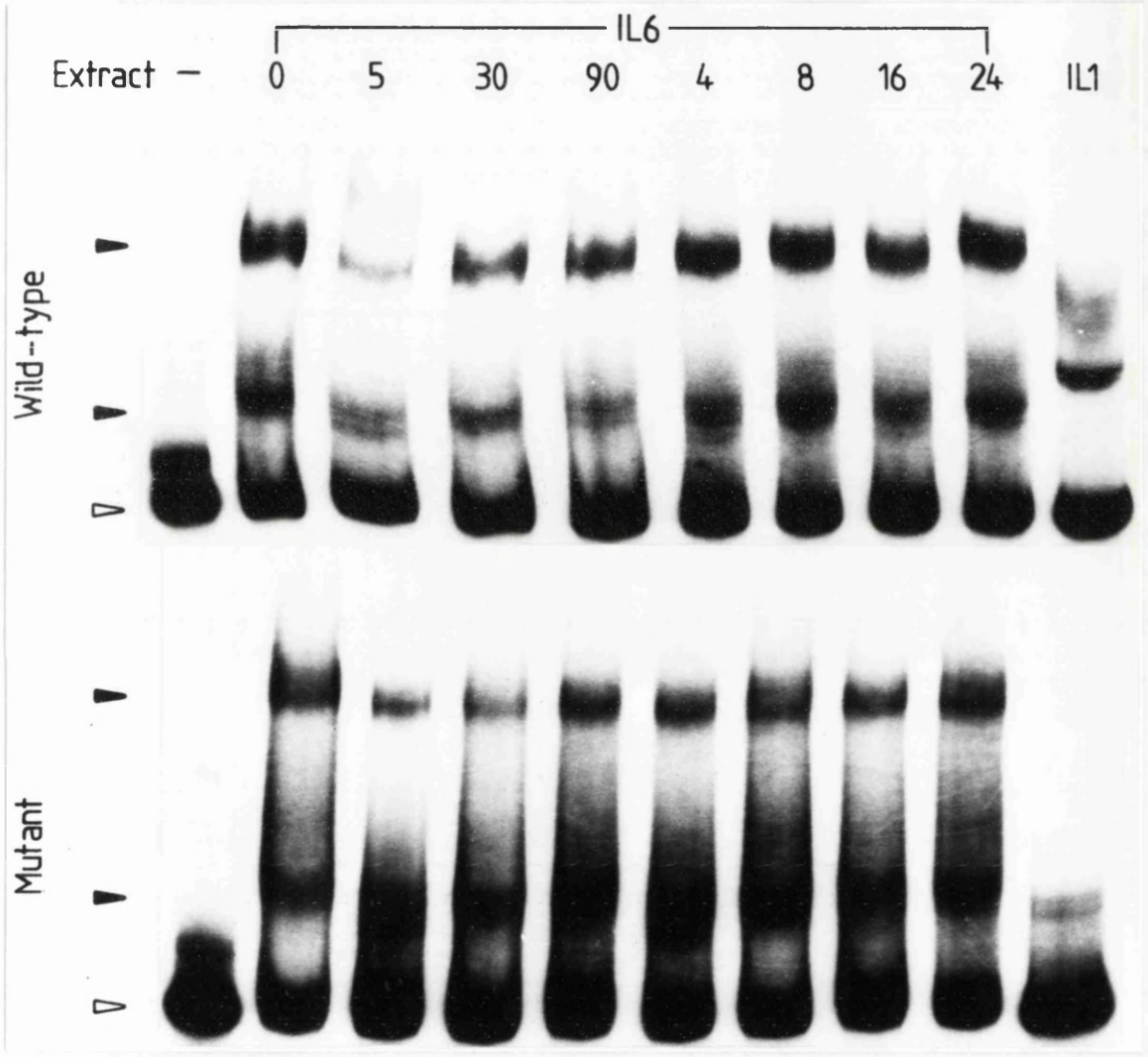


Figure 5.16 Binding of HepG2 Nuclear Proteins to SAA Promoter Fragments in IL6 Time Course.

A) Nuclear proteins isolated from HepG2 cells after treatment with IL6 for the times shown were incubated in bandshift assays with the 265bp fragment containing a wild-type or mutated NFκB site. Open and closed arrowheads = free and shifted bands respectively, IL1 = IL1/HepG2 extract.

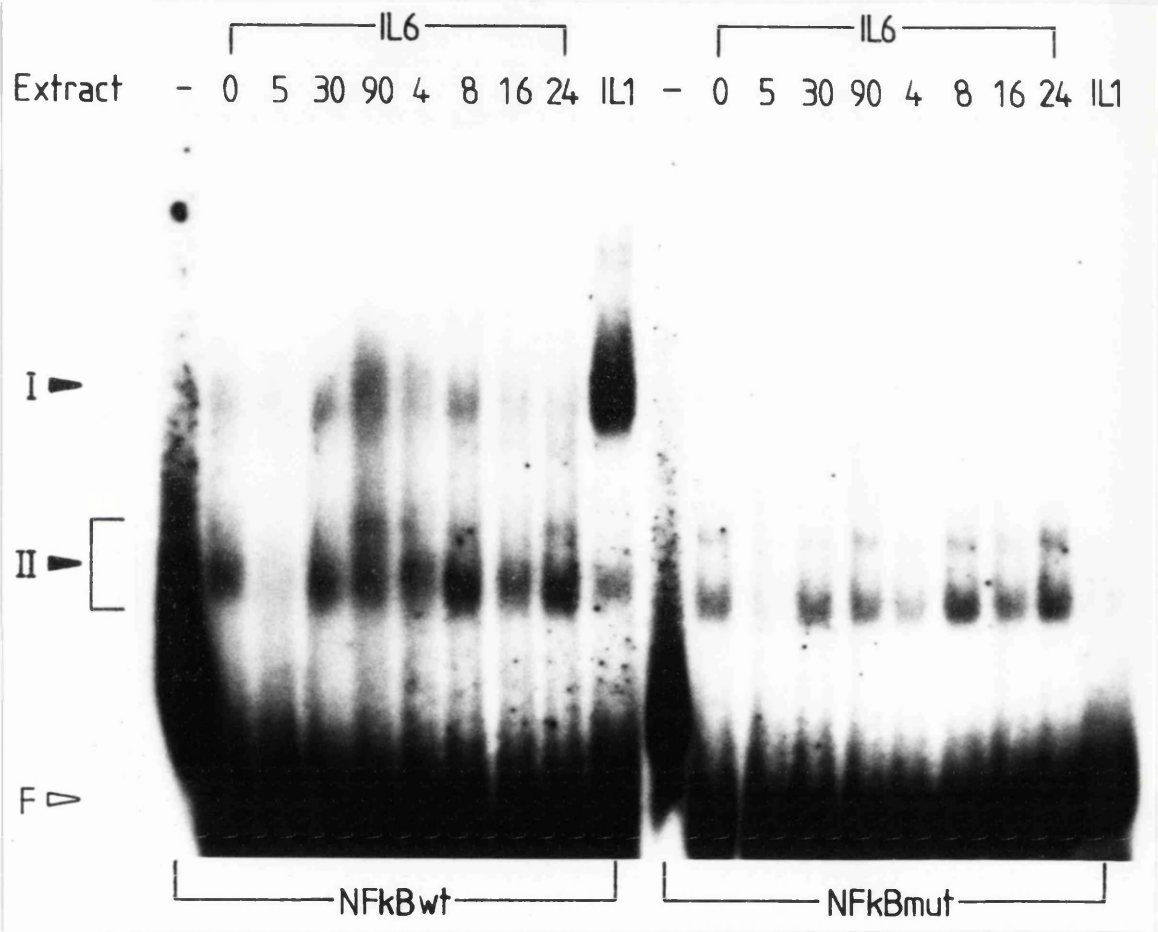


Figure 5.16 (cont.)
B) The same extracts as (A) were incubated with wild-type and mutated SAAκB double stranded oligonucleotide probes as shown. Shifted complexes I and II and free probe F are indicated.

reduced, examination of a methylation interference track run concurrently also revealed disappearance of three Gs in the same region (see figure 5.17). The same footprint was obtained with the first and second sets of IL6 extracts made. The footprinted region encompassed nucleotides -179 to -173 of the *SAA2* 5' region. It was not possible to determine the 5' limit of the binding site however the 3' limit did not include the GGGA of the CTGGGA element thought to be involved in mediating the IL6 induction of acute phase genes. However soon after this binding sequence was found two papers detailing the IL1 induction of the IL6 gene demonstrated the binding site for the transcription factor NFIL6 5'-ACATTGCACAATCT-3' found between -158 and -145 in the human IL6 gene (Isshiki *et al*, 1990; Akira *et al*, 1990). The binding sequence found by footprinting the *SAA2* promoter is homologous and contained the sequence 5'-AGGTTGCACAACTG-3'. Homologies to this sequence were also found in several other acute phase genes and genes involved in the inflammatory response including CRP, haptoglobin, TNFα, G-CSF and haemopexin (Akira *et al*, 1990).

Although protein binding to the NFκB site was observed in all the IL6 extracts no footprint was found in this region, however this region of the gel was often difficult to resolve. The NFIL6-like footprint was obtained from the lower of the two bands seen in bandshift gels using the 265bp fragment, generally the activity eluted from the upper band was too low to obtain readable sequence, factors binding to the NFκB site may have been present in this upper band.

5.2.3.4 Binding of Nuclear Factors to Oligonucleotides Carrying the NF-IL6 Region.

The demonstrated binding region was homologous to the NFIL6 site but it was not possible to demonstrate whether the binding seen was NFIL6 *per se* or a related molecule. Double stranded oligonucleotides were made corresponding to the NFIL6 site in the IL6 promoter, the homologous region in the *SAA2* promoter (IL6RE) and a mutant IL6RE with several bases altered in the binding region (mIL6RE; see section 2.1.5. for sequences of oligonucleotides). When these fragments were labelled, incubated with the IL6 nuclear extracts and bandshift gels run, binding was seen to the NFIL6 and IL6RE sequences but not to mIL6RE (figure 5.18). This analysis showed increased binding intensity at 4 and 8 hours for the IL6 time course. Thus three distinct bands were seen, the upper two being present in all extracts apart from the 5 minute extract when the upper band was absent and a higher mobility one appeared. This band was also visible in the 4 hour extract. This suggested alteration in the status of nuclear proteins binding at the IL6RE following IL6 treatment of HepG2 cells and indicated that they were NFIL6-like in their ability to bind the cognate NFIL6 site but not mNFIL6 whose sequence was based on NFIL6 site mutants which could not bind rNFIL6 (Akira *et al*, 1990). As NFIL6 is a member of the C/EBP family the different bands observed may represent various possible homo- and/or heterodimer forms. Alternativley secondary protein-protein complexes maybe formed in the extracts. Further definition of the alteration in binding specificity at the NFIL6 site

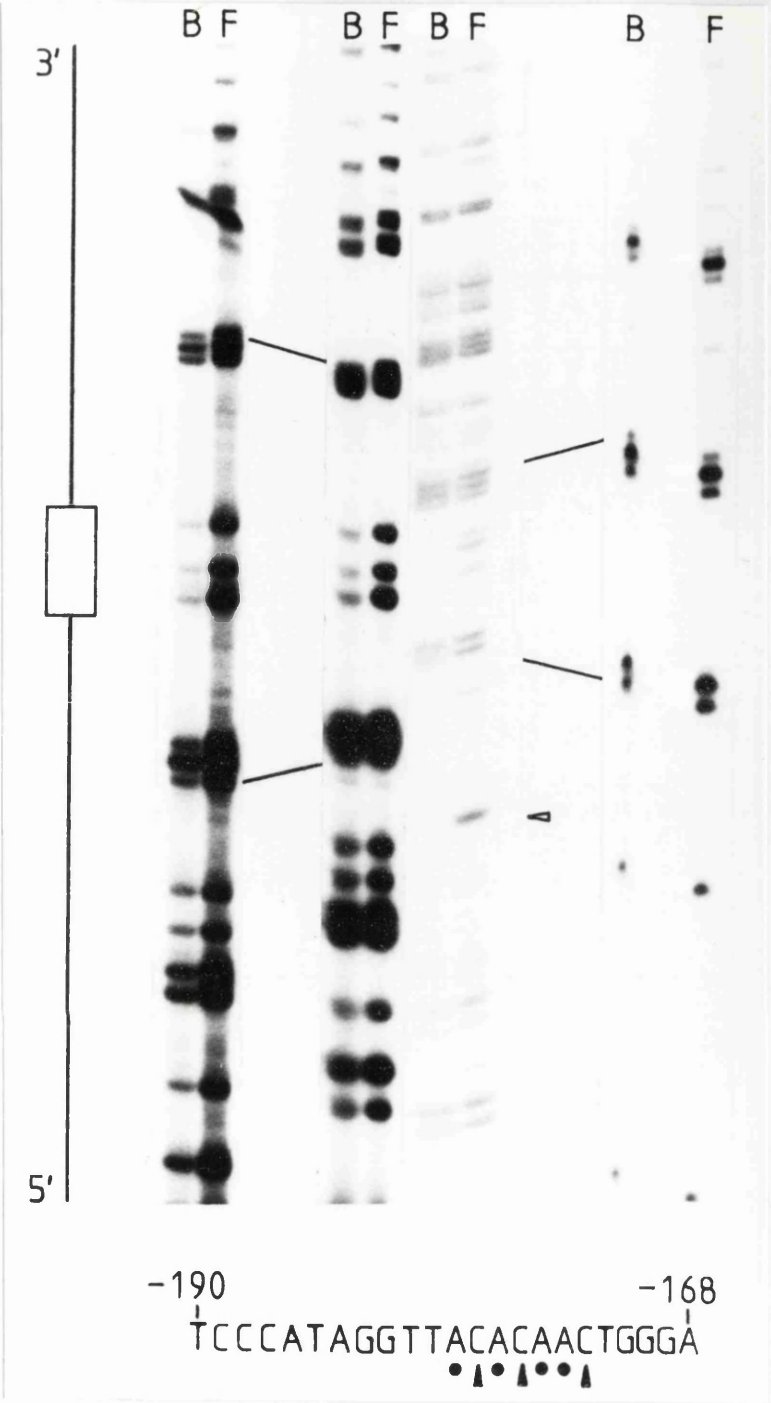


Figure 5.17 Footprint Analysis of Nuclear Factors from IL6 Treated HepG2 Cells Binding to the SAA 265bp Fragment.

Examples are shown of interference of nuclear factors binding from HepG2 cells treated with IL6 for 8hr. Methylation of G residues or modification of C and T residues interfered with binding in the region boxed. The 5' and 3' indicate the orientation with respect to the SAA coding strand. Lanes 1-4 = G-specific chemistry for the lower strand; lanes 5 & 6 = C+T-specific chemistry for the lower strand; lanes 7 & 8 = G-specific chemistry for the upper strand. B = bound, F= free fragment. Sequence between -190 and -168 of *SAA2* is shown at the bottom with bases necessary for binding on the lower strand indicated (arrowhead for G, circle for C). No interference footprint was seen on the upper strand. Lines between the tracks join the same residues. Open arrowhead next to the C+T track denotes spurious band not present in the sequence.

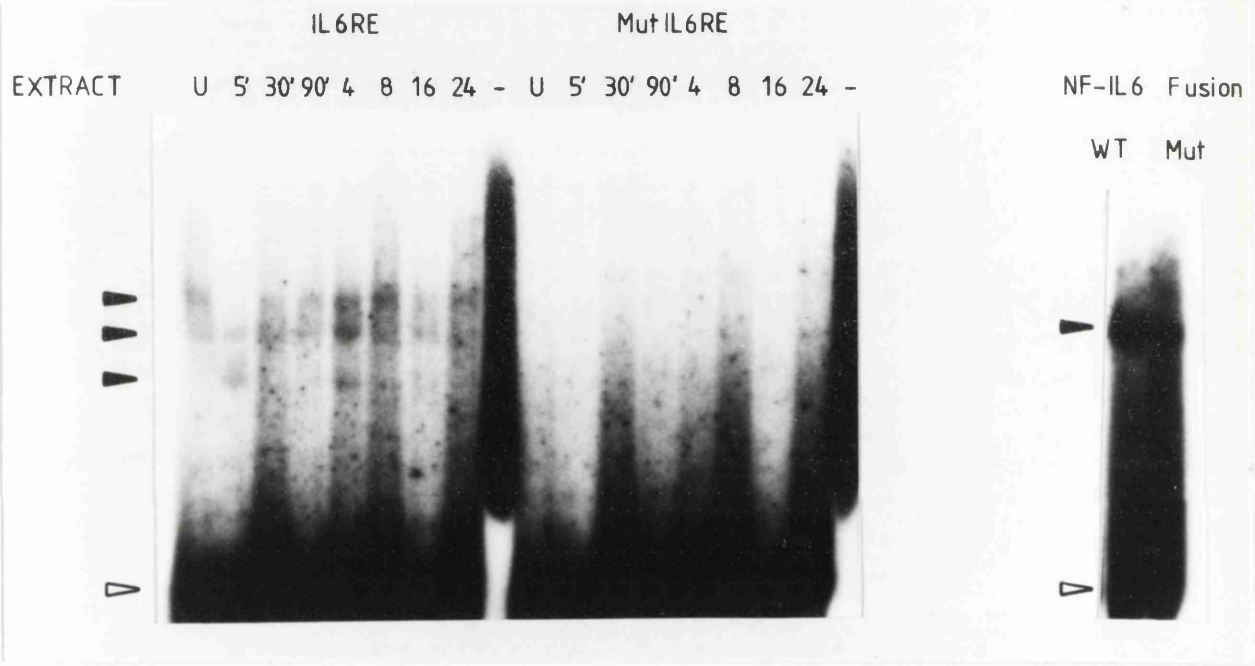


Figure 5.18 Nuclear Factors from IL6 Treated HepG2 Cells Bind Oligonucleotides to the NF-IL6 Region.
Bandshift assays were carried out using wild-type and mutant IL6RE oligonucleotides and the IL6 extracts shown (U = uninduced, - = no extract). The same probes were also incubated with recombinant NF-IL6 fusion protein (right).

awaits detailed footprint analysis comparing untreated and IL6 treated HepG2 nuclear extracts as well as the use of specific antibodies.

A rNFIL6 fusion protein (gift of T. Kishimoto) bound the NFIL6 site and the IL6RE but a reduced level of binding was evident with mIL6RE (figure 5.18). Thus NFIL6 itself can bind *SAA2* promoter sequences, strongly implicating this *trans*-activator or closely related proteins in control of SAA transcription.

CHAPTER 6.

Discussion of Gene Expression Studies.

6.1 *SAA1* and *SAA2* Show Similar Transcriptional Responses in Hepatoma Cells.

Sequence analysis of the 5' flanking region of *SAA1* demonstrated 88% homology with *SAA2* in the 450bp immediately upstream of exon 1. This region contains several potential *cis*-acting sequences (see section 5.2). When this region from *SAA1* and *SAA2* was placed upstream of a CAT reporter gene and transiently transfected into HepG2 cells, stimulation with IL1 and IL6 produced comparable transcriptional responses (figure 5.4). Thus at the functional level as well as the structural level there is clear analogy with the murine system where *SAA1* and *SAA2* respond in a similar fashion to inflammatory stimuli whereas there are differences in the kinetics (Lowell *et al*, 1986b) and tissue specificity (Rokita *et al*, 1987; Brissette *et al*, 1989) of *SAA3* induction dependent on the inflammatory stimulus. The responses of the *SAA1*- and *SAA2CAT* genes were specific as demonstrated by the inability of IL1 or IL6 to increase expression of the TKCAT vector (figure 5.9). In addition all cytokines were tested for specificity using neutralizing antisera, demonstrating that the responses were not due to contaminating cytokines or endotoxin (figure 5.9).

Extensive studies have not yet been carried out to determine whether the non-homologous regions upstream of -450 in *SAA1* and *SAA2* caused a significant disparity in transcriptional activity. In particular *SAA1* did not contain a second NFκB site ($NF\kappa B_2$) found in *SAA2* between -635 and -626, which has been shown to influence transcriptional activity (Woo *et al*, 1991; Edbrooke *et al*, 1991). However a possible NFκB site has recently been identified within the B element homology (fig. 6.1) which contains the sequence AGGGCTTTTCT. Unlike $SAA\kappa B_2$ this was in the same orientation as $SAA\kappa B_1$ with respect to the gene. The B region in *SAA2* was degenerate within this sequence and is unlikely to bind NFκB. Binding of proteins to the putative $SAA1\kappa B_2$ site and functional involvement remain to be demonstrated.

SAA1

CTATTTCCGAATTGACTAGGCCAGCTGAGCA**GGGCTTTTCT**GTGCTGAGGA

SAA2

GGCAGCAGAAAGTCCCCCTCTCTACATTGTCCTTGGCTCAGGAGCCAACTTA GAAAAAGC

Figure 6.1 Sequence of the *SAA1* B Region.
The sequence of the *SAA1* B region as defined in figure 5.1 is shown with a potential C/EBP site underlined and NFκB site in bold. A similar pattern of motifs is found in the 5' flanking region of *SAA2*; $SAA2\kappa B_2$ is in the reverse orientation with respect to the gene and binds an IL1-inducible factor (Edbrooke *et al*, 1991). There are also a possible C/EBP motifs (underlined) in the same relative orientation to the NFκB site as *SAA1*.

The longer *SAA1CAT* constructs (*SAA1CAT*800 and 1600) gave levels of induction in the same range as *SAA1CAT*440 (figure 5.7). Although the basal level of the former two constructs was lower a stepwise increase between two adjacent constructs was not demonstrable as seen for *SAA2* (Edbrooke *et al*, 1991). Further systematic studies using finer deletions of *SAA1* will be required to compare these regions in detail.

Although similar transcriptional responses for *SAA1* and *SAA2* were measured here, the mRNA levels in the acute phase liver of a single individual hybridized with *SAA1* and *SAA2* specific oligonucleotides demonstrated a two-fold higher level of *SAA1* mRNA over *SAA2* (Kluve-beckerman *et al*, 1991b). Studies of human SAA plasma levels have also indicated that SAA1 is the predominant isoform present during the APR (Strachan *et al*, 1986). Clearly these differences may not be due to differing transcriptional responses but could relate to mRNA or protein processing. In this respect the 25bp insertion in the 3' untranslated region of *SAA2* (figure 3.8) maybe important. Sequences at the 3' ends of several cytokine genes have been shown to affect mRNA stability with specific proteins binding to them (Malter, 1989). In addition although the system in the present study was well defined in terms of the cytokines used it is likely that *in vivo* further cytokines are operating which may modulate induction of the two genes differentially. Interestingly despite the strong homology of *mSAA1* and *mSAA2* and comparable expression in the liver, extrahepatic expression can be disparate. For instance the small and large intestines of Balb/c mouse express *SAA1* mRNA but not *SAA2* (Meek *et al*, 1989). Two of the dog SAA genes which had highly homologous cDNAs showed widely differing mRNA induction in the liver following an inflammatory stimulus (Sellar *et al*, 1991).

With the above mentioned exceptions patterns of expression appear to be conserved in the other species studied, for example a probe specific to *ShSAA3* demonstrated that extrahepatic expression was largely due to this gene (Webb *et al*, 1989). The mink *SAA1* and *SAA2* genes showed the same tissue specific pattern of expression although hybridizing to different molecular weight mRNA species (Marhaug *et al*, 1990). Finally in the rat *SAA1/SAA2*-related gene expression was limited to the liver following LPS injection whereas *SAA3* expression was found extrahepatically but not within the liver (Meek and Benditt, 1989b).

Analysis of expression of human *SAA3* has yet to be reported. Sequences in the 5' flanking region bear little resemblance to *SAA1* and *SAA2* (see chapter 3) suggesting that expression may also be divergent. In particular an identifiable NFκB motif is lacking although an AP-1 homology is present. Upregulation of members of the *jun-fos* family by IL1 in various cell types has been shown (Colotta *et al*, 1988; Muegge *et al*, 1990) thus the *SAA3* AP-1 site maybe involved in mediating an IL1 response. The analysis of RNA in cell lines from a number of tissue sources using gene specific probes will enable the identification of tissue specific differences in expression within the human SAA gene family.

It is notable that the transfection studies reported here were carried out in HepG2 cells, a cell line in which the endogenous SAA genes do not respond to cytokines as assayed by Northern analysis (data not shown). Other studies have also failed to detect SAA gene products in these cells (Baumann *et al*, 1984). The transfection experiments demonstrated that the signal transduction and transcriptional machinery required for SAA expression in these cells was intact. Southern analysis of HepG2 cell DNA carried out in the course of this work showed that the SAA genes were present and had not been deleted, although minor deletions might have occurred which affect expression. Potentially the SAA mRNA maybe particularly unstable in these cells or chromatin conformation maybe such that it does not allow the access of transcription factors. Antiquera *et al* (1990) have reported methylation associated gene inactivation (MAGI) occurring in non-essential genes in mouse tissue culture cells. This phenomenon described as an "epimutation" may have occurred in the HepG2 SAA locus where the gene products have not been essential for the survival of cells over time. Presumably this process would be relatively random among non-essentail genes as several AP genes are expressed in HepG2 cells.

The major site of SAA production is the liver, in recent years major insights have been gained into the mechanisms of tissue specific gene expression through the transfection of promoter fragments into different cell types. Studies on liver specific genes such as albumin, transthyretin and α_1AT have led to the identification of important *cis*-acting sequences and *trans*-acting factors. Such proteins include the hepatocyte nuclear factors, HNF1, 2, 3, and 4 (Frain *et al*, 1989; Nicosia *et al*, 1990; Rangan and Das, 1990; Lai *et al*, 1990; Costa *et al*, 1989), members of the liver-enriched CCAAT/enhancer binding protein (C/EBP) family (Graves *et al*, 1986; Johnson *et al*, 1987) including liver activator protein (LAP; Descombes *et al*, 1990) and DBP which recognizes the D site in the albumin promoter (Mueller *et al*, 1990); as well as other factors such as LFA1, LFA2 (Hardon *et al*, 1988; Monaci *et al*, 1988), and members of the CTF/NF1 family (Paonessa *et al*, 1988) . Although these factors were originally identified as liver-specific or liver-enriched, the same or closely related proteins maybe found in other tissues, for example Ig/EBP1 which binds the IgH enhancer and promoter, an homologue of C/EBP is found ubiquitously (Roman *et al*, 1990). Indeed C/EBP itself is found extrahepatically, particularly in adipocytes (Christy *et al*, 1989).

Liver-specific genes often contain multiple binding sites for liver-enriched nuclear factors, for example *M. domesticus* α_1AT has HNF1, HNF3 and C/EBP sites, natural point mutations occurring in these sites in *M. caroli* produces some deregulation of liver specificity (Latimer *et al*, 1990). Examination of the *SAA1* and *SAA2* promoters reveals no obvious liver specific elements. Potentially the NF-IL6 site maybe one component providing a means for generating liver specificity if C/EBP *per se* can bind this site. Alternatively sites maybe distant from the gene promoter or previously unidentified or cryptic sites maybe present. HNF1 which is necessary for CRP gene expression binds to

sites which are degenerate compared to previously known HNF1 sites (Toniatti *et al*, 1990).

An alternative/additional possibility for generating liver specificity is that dominant negative factors are present in non-liver cells which prevent expression there. Studies on the murine *SAA3* gene suggested that this maybe the case. The 5' flanking region of *mSAA3* to -306bp fused to CAT was expressed and induced by CM, IL1 and TNF on transfection into a liver-derived cell line (Hep3B) but not in HeLa. A cytokine responsive element (ILRE) was identified between -185 and -118 which when placed upstream of a cytokine unresponsive minimal promoter (-63bp of *SAA3* fused to *CAT*) conferred CM responsiveness when present in single or multiple copies in Hep3B. These latter constructs were also inducible in HeLa cells leading to the conclusion that the deleted region (-117 to -64) contained elements that conferred repression in non-liver cells (Huang *et al*, 1990). However in a later study the same workers found a C/EBP binding sequence in this region (Li *et al*, 1990) which would presumably operate as a positive liver-specific factor. Perhaps this factor acts synergistically with factors at the ILRE to induce transcription in liver cells whereas deletion of this region in non-liver cells brings the cytokine responsive elements into closer proximity and possibly allows better interaction with the transcriptional machinery. In the context of the full length promoter C/EBP would not be available and cytokine induced factors would have a minimal effect on transcription.

6.2 SAA Transcription is Induced Synergistically by Combinations of Cytokines.

Induction of *SAACAT* genes following transfection into HepG2 cells by combinations of cytokines was greater than the sum of the actions of the individual cytokines and thus synergistic. This was true for combinations of TNFα with IL1 or IL6 but under the conditions used was most marked for IL1 with IL6. *In vivo* hepatocytes would experience combinations of cytokines during inflammation and several groups have studied their interactive effects on production of individual APPs. Combinations of IL1, IL6 and TNF acted synergistically in increasing AGP and decreasing albumin in the rat hepatoma line Fao, whereas the IL6 induction of ßFBN was inhibited by IL1 or TNF (Andus *et al*, 1988a). In HepG2 cells IFNγ and IL1 synergistically induced C1INH protein secretion but the IL6 induction was antagonized by IL1, as was that for FBN (Zuraw and Lotz, 1990). However in mice IL1 and TNF were synergistic in their induction of FBN (Mortensen *et al*, 1988). In cells of human origin IL1 and IL6 synergistically induced both SAA and CRP in Hep3B but only gave the same level as IL6 alone in PLC/PRF/5 where IL1 had no effect alone (Ganapathi *et al*, 1988a). An interesting mechanism for the interaction of IL1 and IL6 induced pathways in the synergistic induction of CRP-CAT fusion genes in Hep3B cells was observed. Although CAT was synergistically induced by IL1 with IL6, S1 analysis of the mRNA showed that IL1 did not enhance the level produced by IL6 therefore IL1 must be acting

translationally (Ganter *et al*, 1989). Presumably this would not be non-specific but involve the first 15nt of CRP which were present in the mRNA.

Synergy between cytokines has been observed in systems other than the APR. Most notably IL1 and IL6 synergize in activating T cells to proliferate and produce IL2 (Helle *et al*, 1988; Houssiau *et al*, 1988; Elias *et al*, 1989a; Mizutani *et al*, 1989; Holsti *et al*, 1989) as well as in B cell differentiation (Emilie et al, 1990). IL1 was also found to be synergistic with a B cell derived activity, CD23 in inducing thymocyte proliferation (Mossalayi *et al*, 1990). Also within the regulatory network of cytokine-cytokine interactions IL1 synergizes with TNF in the stimulation of fibroblast IL1 (Elias *et al*, 1989b) and IL6 production (Elias and Lentz, 1990).

As previously described IFNγ is synergistic with TNF in its antitumour action on many cell lines (Ruggiero *et al*, 1986; Esparza *et al*, 1987) and in induction of release of CSFs by monocytes (Lu *et al*, 1988). These effects appear to be mediated at least in part by the IFNγ induced upregulation of TNF-R (Aggarwal *et al*, 1985; Ruggiero *et al*, 1986). IFNγ also synergizes with IFNα, IFNß and IL2 (Czarniecki *et al*, 1984; Svedersky *et al*, 1984) as well as IL1 (Chen *et al*, 1987).

Thus the synergistic action of combinations of cytokines appears to be a general mechanism for controlling cellular responses and has clear advantages in terms of economy and rapidity of response. Despite its widespread occurrence the major mechanisms by which synergy occurs has not been elucidated in many systems. Experiments were therefore carried out using the SAA system as a model to investigate one aspect of IL1/IL6 synergism (see section 6.4).

6.3 NFκB-like and C/EBP-like Proteins Play a Central Role in Mediating Acute Phase Gene Transcription.

6.3.1 Involvement of NFκB-like Proteins.

In order to address the mechanism of IL1/IL6 synergism, the actions of the individual cytokines were first considered. One pathway of IL1 action leads to the activation of the transcription factor NFκB. Indeed mutation of the NFκB enhancer sequence GGGACTTTCC in the *SAA2* promoter to CTCACTTTCC, a mutation which abolishes NFκB binding (Nabel and Baltimore, 1987) abolishes IL1 induction of SAACAT (see figure 5.10). The involvement of NFκB-like proteins in the IL1 induction of other AP genes has also been shown. The rat angiotensinogen gene has an acute phase response element (APRE) between -552 and -537 which binds both an IL1-inducible 50kD NFκB-like protein and a constitutive 32kD protein (Ron *et al*, 1990). In the mouse factor B gene an NFκB motif was identifed within a region which when deleted (-553 to -478) abrogated IL1 induction. Subsequent mutation of the NFκB sequence resulted in loss of binding of an IL1 inducible factorin HepG2 cells and failure to transactivate a minimal factor B promoter (Nonaka and Huang, 1990). The NFκB site in the SAA promoter in addition to acting as an IL1 inducible enhancer is also an essential promoter

element as disruption of NFκB binding drastically reduces the basal level of transcription. Thus constitutive levels of NFκB proteins are essential for transcription from the SAA promoter and as such can be viewed as part of the transcriptional machinery of this gene.

6.3.2 NF-IL6, CTGGGA and IL6 Induction.

The mechanism by which IL6 activated the SAA promoter was unknown. The induction of an SAA promoter containing a mutated NFκB site by IL6 was reduced compared to wild-type. However there was still a residual level of expression (see figure 5.8) indicating that other sequences were involved. The hexanucleotide sequence CTGGGA was found between -173 and -168, sequences similar to this have been found in a number of other AP genes including rat FBN, α_2M, AGP and kininogen, *mSAA3* and human CRP, Hp and α_1AT (see table 6.1). This element originally found within homologous regions of the rat FBN genes suggested that they may provide a basis for the coordinate regulation of these genes (Fowlkes *et al*, 1984). Despite the finding of CTGGGA-related elements in AP genes evidence for direct involvement as an IL6 responsive element has remained equivocal. Kunz *et al* (1989) deleted the region between -404 and -165 of the rat α_2M gene which contained a CTGGGA element and found that IL6 activation of the transfected gene in HepG2 cells was abrogated. Direct mutation of this sequence to GATATC resulted in a 60% loss in IL6 inducibilty (Hattori *et al*, 1990). Rat liver nuclear proteins bound across this sequence, while a promoter fragment spanning -209 to -130 showed altered mobility in band shifts of nuclear proteins after IL6 treatment of rat hepatoma cells. This latter complex could be competed by oligonucleotides containing CTGGGA from AGP and *mSAA3* promoters (Hattori *et al*, 1990). However in all these cases the elements identified contained potential NF-IL6-like sequences (see below). For example mutation of the CTGGGG sequence in the human C3 gene promoter to GATATC led to loss of IL1 and IL6 inducibility of an heterologous gene in Hep3B cells. However nuclear protein binding to a C3 promoter fragment containing this element could be competed by the mutated sequence (Wilson *et al*, 1990) and the mutated bases overlap with a C/EBP/NF-IL6 binding site which may account for the lack of inducibility. Thus the conclusion that CTGGGG binds an IL6 responsive factor (Wilson *et al*, 1990) requires corroboration by further fine detail mutation analysis to demonstrate a distinct binding element. In the human CRP gene, mutation of the CTGGGA-related element had little effect on induction in Hep3B cells by MoCM whereas mutation of a sequence just downstream in the ß subregion of acute phase response element 1 (APRE 1) caused a total loss of inducibility (Majello *et al*, 1990).

Studies on the human *SAA2* promoter described in chapter 5 revealed a similar situation. Deletion to -100 created a construct (*SAA2CAT*100) which contained the NFκB site but lacked the CTGGGA element between -173 and -168. Although the PstI site used to clone *SAA2CAT*100 meant that the SAA promoter region terminated only 5bp upstream of the NFκB motif, this was fully functional as IL1 inducibility was maintained (figure 5.10). However Il6 inducibility of *SAA2CAT*100 was significantly reduced

compared to *SAA2CAT*440, this was particularly notable after 16 hours after stimulation where the level of transcription of *SAA2CAT*100 was below its own constitutive level (see below for possible explanation of this effect). Thus sequences between -400 and -100bp were important for mediating the IL6 response.

Table 6.1 CTGGGA-Related Elements in AP Genes.

Gene	Sequence	Ref.
HuSAA1/2	-175 AACTGGGAT -167	1
MoSAA1/2	-138 CACCGGGAA -130	2
	-222 AACTGGGGC -230	2
MoSAA3	-82 AAGTGGGAT -74	2
	-61 AACAGGGAT -53	2
Rat FBNα	-131 TTCTGGGAA -123	3
Rat FBNß	-152 TGCTGGGAA -144	3
Rat FBNγ	-153 ATCTGGGAA -145	3
Rat α_2M	-166 TTCTGGGAA -158	4
Rat AGP DRE	82 TTCTGGGAA 90	5
Hu α_1AT	-122 GGCTGGGAT -130	6
Hu CRP	-77 TGTTGGAAA -69	7
Hu C3	-103 ATCTGGGGC -95	8
Hu Hp	-127 TACTGGAAA -119	9

References. 1, present study; 2, Lowell *et al.* (1986b); 3, Fowlkes *et al.* (1984); 4, Hattori *et al.* (1990); 5, Won and Baumann (1990); 6, Monaci *et al.* (1988); 7, Majello *et al.* (1990); 8, Wilson *et al.* (1990); 9, Oliviero *et al.* (1987).

To obtain further information with respect to possible sequences involved in IL6 induction, nuclear extracts were made from IL6 treated HepG2 cells over a time course of 24 hours. These extracts contained proteins which bound specifically to a 265bp fragment of the SAA promoter from -233bp to +32bp. with increased binding at 8 and 16 hours (see figure 5.12). The same pattern of binding was also observed with the 265bp fragment containing a mutated NFκB site. Although binding occurred to double stranded oligonucleotides containing the SAAκB region, binding also occurred to other upstream regions as shown by bandshifts with fragments such as the PstI fragment (figure 5.15). Methylation interference footprinting demonstrated the involvement of three guanine residues on the antisense strand at positions -173, -176 and -178 in interacting with a nuclear factor (figure 5.17). These residues were later found to be within a sequence homologous to the binding site in the IL6 gene for NF-IL6 (Isshiki *et al*, 1990; Akira *et al*, 1990). Like the C3 gene this element is adjacent to the CTGGGA element, however there was no evidence for involvement of the 3 Gs of this sequence in factor binding from methylation interference analysis or via chemical modification of C and T nucleotides on

the antisense strand and subsequent interference with binding (figure 5.17). However footprinting analysis, for example using DNAse I protection or phenanthroline copper footprinting will be required to define the boundaries of the binding sequence more clearly.

Binding of proteins to this region was also confirmed by using double stranded oligonucleotides extending from -188 to -166 of *SAA2* as probes in bandshift assays which bound factors in both constitutive and IL6 induced nuclear extracts (figure 5.18). However when the sequence CCATAGGTTACACAACTGGGATA was mutated to CCATAGGCTACAAACCTGGGATA binding of proteins from these extracts was diminished. Factors in these extracts also bound to the cognate NF-IL6 recognition site as present in the IL6 gene (not shown) and recombinant NF-IL6 fusion protein bound to the IL6RE oligonucleotides. These data provide evidence that the factor(s) binding at the IL6RE are members of the C/EBP/NF-IL6 family. Site directed mutagenesis of the NF-IL6 enhancer-like region in *SAA/CAT* genes will provide a functional test to link the DNA binding observed to IL6 induction.

Studies in a number of laboratories have demonstrated IL6 induction and binding of liver cell derived nuclear proteins to acute phase gene promoters. Many of the results obtained were similar in that multiple binding entities were observed in nuclear extracts which showed some alteration in binding on IL6 treatment. However no obvious homologous sequences were found between the various genes. Reexamination of this data in the light of the work of Isshiki *et al* (1990) and Akira *et al* (1990) suggests the possible involvement of NF-IL6-like proteins in many of these cases. Mutations in the human CRP promoter α and ß regions reduced IL6 and MoCM inducibility, a strong homology to the NF-IL6 site (table 6.2) is present in the α region as well as other possible sites. An oligonucleotide to the α region (OLα) bound factors in uninduced Hep3B nuclear extracts, with further retarded bands occurring following IL6 or MoCM treatment of cells (Majello *et al*, 1990). The HxA site and the HpA and C sites could all compete with varying efficiency for factor binding to OLα (Majello *et al*, 1990). These sequences also contain NF-IL6 homologies (table 6.2). Three regions with enhancer activity have been identified in the human Hp gene promoter: A (-186 to -156), B (-153 to -87) and C (-90 to +1). The A and C subregions show similar binding patterns in bandshifts with Hep3B nuclear proteins with an extra band appearing after IL6 treatment. The B subregion has a distinct binding pattern which does not alter after IL6 induction. Footprinting of A and C showed patterns of protection in regions with homology to NF-IL6 sites (see homologies in table 6.2) and these fragments can confer IL6 responsiveness on a SV40 promoter (Oliviero and Cortese, 1989). In the rat haptoglobin gene a region homologous to the human Hp promoter was found, but only a small IL6 response was achieved in transfected cells. A point mutation (A to G at -160) made the sequence identical to the human sequence and increased the IL6 responsiveness by 2-3 fold (Marinkovic and Baumann, 1990). This mutation was within the NF-IL6 enhancer-like region.

Similar results were obtained for the Hx gene with an inducible protein (IL6DBP) appearing after treatment of Hep3B cells with IL6 (Poli and Cortese, 1989). Again the footprint in this region involved a sequence with homology to the NF-IL6 site. Subsequent cloning of rat IL6DBP confirmed that this was a member of the NF-IL6 family (see below). Involvement of NF-IL6 in rat AGP expression is also likely as the A and B elements of the distal response element (DRE) together can respond to IL1 but when an 8bp sequence is inserted between fragments A and B this is lost (Won and Baumann, 1990). The junction of the A and B fragments falls within an NF-IL6 binding site homology.

Table 6.2 NF-IL6 Homologous Regions in AP Genes.

Gene	Sequence	Ref.
Hu IL6	-158 ACATTGCACAATCTTAA -142	1
HuSAA1	-184 AGGTTGCACAACTGGGA -168	2
HuSAA2	-184 AGGTTACACAACTGGGA -168	2
MoSAA1/2	-186 CCATTGCACAATGAGGG -170	3
MoSAA3 ILRE	-183 GACTTTCTGAAGTCTTC -199	3
MoSAA3 PRI	-56 GGATTGCTCCATTAGTG -40	4
MoSAA3 PRII	-77 GGATTATGCAAGATCAA -61	4
MoAGP	-117 ATTTTGCGCAAGACATT -101	5
	-105 ACATTTCCCAAGTGCTG -89	5
	-72 CTGTGGCACAATCTCAC -88	5
Rat FBNß	-142 ATGTTGCTCAAATGATA -126	6
Rat α_2M	-155 GAATTCCCAGAAGGATT -171	7
Rat AGP DRE/A	9 ACATTTCTTAATCTTCC 25	8
Rat AGP DRE/A/B	25 CCATTGCACAACTTAGG 41	8
Rat Angioten.	-548 GGATTTCCCAACCTGAC -532	9
Rat Hp	-162 GTATGAAGCAAGAGCTC -146	10
Hu HpA	-169 GTGTGAAGCAAGAGCTC -153	11
Hu HpC	-71 GAATTACGAAATGGAGG -55	11
Hu HxA	-105 TGATTACATCACTGCA -121	12
Hu CRPα	-45 AGTTTGCGCCACTATGT -61	13
Hu C3	-113 CCATTTCCTAAGCTTTT -129	14
	-100 AGATTTCTCAATACCAT -116	14

Sequences which are homologous to the NF-IL6 binding site in the promoter regions of AP genes are aligned. Inclusion of sequences is based on both primary sequence homology and examination of functional data as described in the text. Nucleotide numbering of the rat AGP DRE sequences is based on the DRE in isolation which is approximately 5kb upstream of the transcription start site. References: 1, Isshiki *et al.* (1990); 2, present study; 3, Lowell *et al.* (1986b); 4, Li *et al.* (1990); 5, Chang *et al.* (1990); 6, Fowlkes *et al.* (1984); 7, Hattori *et al.* (1990); 8, Won and Baumann (1990); 9, Brasier *et al.* (1990); 10, Marinkovic and Baumann (1990); 11, Oliviero and Cortese (1989); 12, Poli and Cortese (1989); 13, Majello *et al.* (1990); 14, Wilson *et al.* (1990)

Evidence for the involvement of NF-IL6 proteins in the genes discussed above has now been confirmed in several cases. Poli *et al* (1990) found that recombinant C/EBP bound the HxA, HpA, HpC and CRPα site oligonucleotides, a process which was inhibited by the addition of a synthetic peptide containing the C/EBP leucine zipper sequence (presumably because the peptide bound leucine zippers of intact molecules preventing dimerization and DNA binding). Multimerized HpC was used to probe a a rat λgt11 library and IL6DBP isolated, a protein with strong homology to C/EBP in the bZIP region but was diverged outside this domain. Antibodies to IL6DBP immunoprecipitated 3 times more protein from Hep3B cells after IL6 treatment than before (Poli *et al*, 1990). Chang *et al* (1990) isolated a C/EBP-like cDNA clone from mouse cells which bound to the mouse AGP promoter, again this protein (AGP/EBP) showed strong homology to C/EBP and was inducible in mouse livers by LPS treatment. C/EBP itself is thought to be important in driving the liver specific expression of the mouse *SAA3* gene with two binding sites in the proximal region (PR) (Li *et al*, 1990). A further C/EBP-like element in an upstrean region (ILRE, table 6.2) maybe responsible for AP regulation of *mSAA3*. C/EBP-like proteins also play a pivotal role in rat angiotensinogen gene regulation (see below).

Thus the demonstration of a nuclear factor binding region of the *SAA* promoter which is potentially involved in IL6 gene regulation which can bind NF-IL6 and related proteins in HepG2 nuclear extracts has been corroborated by the involvement of similar transcription factors and *cis*-acting sequences in the acute phase regulation of a number of genes.

6.3.3 Other Factors Binding in the NFκB Region.

The preliminary studies using IL6 treated HepG2 nuclear extracts showed that factors bound to both the wild-type 265bp fragment as well as the same fragment carrying a mutated NFκB site. This indicated that the observed binding did not involve NFκB *per se*. However this was complicated with the finding that the binding entities could be competed by unlabelled oligonucleotides carrying the SAAκB region, which was confirmed in bandshifts using the oligonucleotide as probe (figures 5.13, 5.14, 5.16). Binding also occurred to oligonucleotides carrying a mutated NFκB sequence thus indicating that proteins were indeed binding to this region but in a manner distinct from NFκB. Using the SAAκB oligonucleotides as probes two major complexes were observed with the IL6 extracts, the lower mobility complex was not apparent when the NFκB sequence was mutated (figure 5.16) suggesting that a low level of an NFκB-like protein was present. The higher mobility shifted complex (complex II, figure 5.16) became more intense when the mutated sequence was used as probe. Thus it appears that the two protein species, the NFκB-like protein and complex II proteins, bind the DNA in a mutually exclusive manner. Mutation of the three Gs in the binding site prevents binding and competition by NFκB-like factors with complex II proteins and allows more effective binding of these latter species. Complex II binding occurs probably occurs 3' to, but

sterically overlapping with, the NFκB site as only a short 5bp region is present 5' of the NFκB site in the probes used (section 2.1.5). Footprinting and mutation of these sequences will enable further characterization of the residues important for binding of the complex II proteins.

A recent report has provided support for these observations in studies of the rat angiotensinogen acute phase response element (APRE). The APRE binds an IL1-inducible NFκB-like protein (BPi) in a manner which excludes binding by constitutive factors (BPc's). A mutated APRE that binds only BPi acts as an IL1 inducible enhancer, whereas a mutated APRE that binds only BPc's is not inducible and acts as a constitutive enhancer (Brasier *et al*, 1990). Antibodies against C/EBP used in this latter study reacted with the BPc's. By analogy the complex II proteins in the HepG2 extracts may belong to the same family, thus the proteins which bind at the IL6RE may also bind the SAAκB region although this sequence is degnerate compared to known binding sites for C/EBP-like factors. Initially it seemed unlikely that the same proteins bound the IL6RE as well as the SAAκB region given the apparent unrelatedness of these sequences. The data of Brasier *et al* (1990) indicate that this is a possibility. Degeneracy in *trans*-acting factor binding sites has been recognised in a number of protein families including the homeobox proteins such as Oct-1 which can bind to a wide array of sequences (Baumruker *et al*, 1988). A broad range of homeobox proteins can bind the AT-rich sequence TCAATTAAAT in the *Drosophila engrailed* promoter including proteins which only have 25% homology in the homeobox, Oct-2 can recognize both this sequence and the cognate octamer sequence (see Levine and Hoey, 1988).

A conundrum is presented by the findings that abrogation of NFκB binding significantly reduces IL6 induction of the SAA promoter in functional studies, yet NFκB does not appear to be induced by IL6 in nuclear extracts. Indeed IL6 may induce (complex II) proteins which compete with NFκB for their sites in the SAAκB region. A plausible explanation of this is that factors binding at the IL6RE together with a basal level of NFκB interact with the transcriptional machinery in a cooperative fashion to activate transcription. Factors which are constitutively present at the IL6RE are replaced by positive *trans*-acting factors on IL6 induction, together with a basal level of NFκB, which on its own activates transcription minimally, induction occurs. Assuming complex II binding in the NFκB region does represent C/EBP-like factors then IL6 may modulate their activity as well. Paradoxically this may not contribute positively to transcription. Brasier *et al* (1990) suggest that the BPc's binding at the angiotensinogen APRE attenuate BPi (NFκB) binding. Indeed when the BPc binding site was mutated but allowing BPi binding, IL1 induction was increased. For SAA such a mechanism awaits confirmation by mutation of the complex II binding site, by analogy with angiotensinogen an increase in IL6 response may occur as binding of NFκB would occur under conditions of relaxed competition. If complex II proteins are induced by IL6 increasing competition with

NFkB-like proteins this may suggest a self-regulating mechanism whereby the prolonged activation of the genes is prevented and the response is limited to a specific time window.

6.4 Transcriptional Synergy.

6.4.1 Mechanisms.

While it is necessary to study the effects of cytokines on a system individually at the outset in order to define mechanisms of action, the ultimate aim is to gain information on the acute phase response *in vivo*. The network of cytokine interactions *in vivo* is undoubtedly complex, in the relatively confined field of acute phase gene expression alone the concerted action of cytokines may not be simple, having additive, synergistic and inhibitory effects (see above and Gauldie, 1989). Combinations of IL1, IL6 and TNF were found to behave synergistically in activating SAA transcription. These observations have been confirmed in a mouse L cell line permanently transfected with human *SAA2β* where combinations of cytokines were found to synergistically stimulate SAA mRNA accumulation (M. R. Edbrooke, unpublished observations). Thus the synergistic activation of transcription appears to go at least some of the way towards explaining the 2-3 orders of magnitude increases seen in SAA plasma levels during the APR. Contributions may also be made by increased mRNA stability and/or increased translation and secretion rates. However increased transcription is probably the major mechanism given the inefficiency of transcription occurring in transient transfection assays. Sequences like enhancers generally stimulate transcription *in vitro* by factors of a few hundred or less, this compares to the 10^8-fold differences in transcription rate of the growth hormone gene during development (see Cook, 1989). It will be possible to address this by measuring transcription through run-on experiments of endogenous or permanently transfected genes. A several hundred-fold increase in the transcription of the murine SAA genes was measured in the liver by such a method (Lowell *et al*, 1986b)

A large number of studies have shown that increasing the number of binding sites for a factor or factors increases transcription to well above the levels expected for the sum of the effects of the individual sequences (for example see Giniger and Ptashne, 1987; Carey *et al*, 1990). However the mechanism by which multiple factors communicate with the transcriptional machinery to synergistically activate transcription is unknown. For example if the factors all have acidic activating regions, presumably they interact with the polymerase complex by the same mechanism, seemingly making synergy unlikely. However it is clear now that there are different classes of transcriptional activating regions, which may interact with polymerase in different ways to promote synergy. Nevertheless activating domains of the same class such as the glutamine-rich domains of Sp1 can activate transcription synergistically (Courey *et al*, 1989).

In some systems synergy is brought about by factors which bind in a cooperative manner to DNA. For example the human progesterone receptor (PR) binds cooperatively to paired responsive elements. Occupation of one PRE site by the PR dimer increases the

binding affinity of the second site by 100-fold (Tsai *et al*, 1989). This is not universal among steroid hormone receptors as oestrogen receptor molecules (ERs) do not bind cooperatively to EREs but do activate transcription synergistically (Ponglikitmongkol *et al*, 1990). However a near-universal finding is that transcriptional synergy is dependent on the distance between enhancer sites and the correct stereoalignment of the factors involved as well as the exact nature of the binding sequence. This is true for steroid hormone receptors (Ponglikitmongkol *et al*, 1990) as well as the cooperative interaction of factors such as Sp1 and Oct-1 (Janson and Petersson, 1990) although there are exceptions (Frampton *et al*, 1990). LeBowitz *et al* (1989) found that the cooperative binding of transcription factors, in the case of Oct-2 binding to adjacent octamer sites required only the POU domain and thus protein-protein interactions may promote synergy.

In many cases protein-protein interactions seem to be an unlikely mechanism for cooperativity, particularly where factors from different organisms are responsible, for example, rat GR and the yeast activator GAL4 cooperatively activate transcription from a promoter containing binding sites for both proteins (Kakidani and Ptashne, 1988). It has also been shown that multiple NFκB sites activate transcription synergistically in the absence of cooperative binding (Pettersson and Schaffner, 1990). Where cooperative binding does not occur it is predicted that synergism should occur under conditions when both activators are present at concentrations sufficient to saturate their binding sites on the DNA - in such a situation cooperative binding is irrelevant. Carey *et al* (1990) and Lin *et al* (1990) showed that transcription was activated synergistically under conditions of saturable binding to multiple sites for GAL4 and by simultaneous binding of heterologous proteins (GAL4 and the mammalian factor ATF) at saturation. These authors propose that synergism occurs by factors simultaneously "touching" the transcriptional machinery, their experiments indicated that as many as 5-10 molecules may touch the target molecule at once.

A further possibility regarding transcriptional synergy is that factors induced by one agonist are limiting but the presence of molecules activated by the second agonist increases the availability of the factor several fold. An interesting example of this has been observed with IFNα stimulated genes (ISG's) which are negligibly induced by IFNγ but the combination of IFNα and IFNγ is synergistic. ISG activation is dependent on the transcription factor ISGF3 which is comprised of two subunits - treatment with IFNγ increases the abundance of one subunit by ten-fold from limiting concentrations allowing subsequent activation by IFNα (Levy *et al*, 1990). This is also an example of "priming" at the level of transcription factors.

6.4.2 SAA Transcription.

Thus synergistic activation of transcription by IL1 and IL6 induced factors at the SAA promoter could occur by one or a combination of mechanisms. Firstly NFκB binding was essential for IL1/IL6 synergism, thus a quantitative increase in NFκB may

account for the increased transcription. However no induction of NFκB by IL6 was seen in nuclear extracts, although NFκB binds DNA as a multimeric complex (Baeuerle and Baltimore 1988a; 1988b; Nolan *et al*, 1991) and thus like IFNγ and ISGF3, IL6 may upregulate one half of the molecule without inducing DNA binding alone. There are other possibilities for multiple pathways acting on a single factor. For example serum response factor (SRF) is activated by distinct signalling pathways, PKC induces protein-protein binding of $p62^{TCF}$ to the SRE-SRF complex whereas SRF that does not bind $p62^{TCF}$ can still activate transcription when stimulated by PKC-independent activators (Graham and Gilman, 1991).

In addition to the requirement for the NFκB site for synergistic induction of transcription by IL1 and IL6, functional studies with *SAA2CAT*100 showed a reduced level of synergism (*i.e.* in the absence of the IL6RE). The absolute level of induction of *SAA2CAT*100 was similar to that for constructs containing the IL6RE with IL1+IL6 but the IL1 induction was much higher, thus the degree of synergy was reduced. The reason for the higher IL1 induction of *SAA2CAT*100 was unclear, indicating the presence of an inhibitory activity upstream of of -100. This construct terminated close to the NFκB site and may have altered secondary structure in this region in a manner that facilitated DNA binding, the secondary structure in the context of the whole promoter being inhibitory. Point mutations of the IL6RE will demonstrate whether factors at the IL6RE, secondary structure or factors binding elsewhere are the cause of this inhibitory activity. The data obtained using IL6 alone suggests that activators binding at the IL6RE could make a contribution to the synergistic effect.

Clearly synergy does still occur in the absence of the IL6RE. Again the problem arises that complex II proteins compete for binding with NFκB, although NFκB is present in the nucleus at much higher levels following stimulation with IL1+IL6 than following IL6 treatment. However this competition may not inhibit transactivation under conditions when the system is presumably geared for maximal transcription. For example in the mouse mammary tumour virus (MMTV) promoter, the steroid hormone response element (HRE) is adjacent to a site for nuclear factor I (NFI). In cells containing low levels of NFI transcription is stimulated weakly by steroid hormones but cotransfection of an NFI cDNA greatly enhanced both progesterone and glucocorticoid stimulation. Far from binding cooperatively to the adjacent HRE and NFI site the respective factors sterically competed for binding (Bruggemeier *et al*, 1990). A similar situation occurs in the rat growth hormone promoter where Pit-1 and Sp1 bind in a mutually exclusive manner but the integrity of both sites is required for maximal activation of the promoter (Schaufele *et al*, 1990). Thus the mutually exclusive binding of complex II and NFκB proteins does not preclude synergistic enhancement of transcription.

It was apparent from time course studies that maximal binding of NFκB induced by IL1 and C/EBP-like proteins by IL6 occur at widely differing time points. NFκB binding occurs within 15 minutes of stimulation and terminates by approximately two

hours (Edbrooke *et al*, 1991). In contrast maximal binding of IL6 induced proteins occurs much later at 8-16 hours in HepG2 cells, an observation which is supported by the 3-fold increase in IL6DBP in rat liver at 12 hours (Poli *et al*, 1990), the increase in binding seen in Hep3B cells following IL6 treatment (Oliviero and Cortese, 1989) and increased NF-IL6 binding in SK-MG-4 glioblastoma cells after 6 hours treatment with IL1 (Isshiki *et al*, 1990). Despite the relatively long time course *de novo* protein synthesis is not required for NF-IL6 (Akira *et al*, 1990) or IL6DBP (Poli *et al*, 1990) induction. The maximal binding activities of IL1 and IL6 induced nuclear proteins do not coincide temporally, however there maybe earlier modifications (*e.g.* phosphorylation, altered protein-protein interactions) or interchange of facors (from a constitutive non-transactivating protein to a positive trans-activator). Such a modification may facilitate synergistic interactions with induced levels of NFκB, with a sustained or enhanced transcriptional response occurring at later time points with increased binding of IL6 induced proteins. Also noteworthy is that in the original studies on NF-IL6 it was IL1 which induced this factor (Isshiki *et al*, 1990; Akira *et al*, 1990), this has yet to be demonstrated in HepG2 cells but raises the possibility that different subunits of NF-IL6-like proteins may be induced by IL1 and IL6 leading to synergistic interactions.

Wilson *et al* (1990) proposed that the adjacent/ overlapping NF-IL6-like IL1 responsive region and the CTGGGA-like element (proposed to be IL6 responsive) in the human C3 promoter bind factors which interact to promote IL1/IL6 synergism in Hep3B cells. However no evidence has been found for nuclear factor binding to CTGGGA in the SAA promoter which is in a similar location with respect to the NF-IL6 site as the C3 gene and at present does not appear to have a role in IL1/IL6 synergism. No direct evidence was presented for binding to CTGGGG by Wilson *et al* (1990) as discussed above, however the differences observed here between the two studies may represent variation between the cell lines used.

6.4.3 A Model for Transcription Factor Interactions at the SAA Promoter.

A model which is consistent with the results obtained thus far can be summarized as follows for the different stimulatory states studied at the SAA promoter.

i) Constitutive: C/EBP-like proteins are bound at the IL6RE and the NFκB region, these may or may not contribute to the low level of basal expression occurring in transfected cells. However this basal expression is clearly dependent on an NFκB-like protein as abrogation of binding drastically reduces constitutive transcription.

ii) IL1: NFκB is massively induced and effectively competes binding of constitutive C/EBP-like (factors) to activate transcription.

iii) IL6: modification of C/EBP-like factors occurs, these interact synergistically with the basal level of NFκB to induce transcription. The alteration in abundance of one of the subunits of the constitutively bound dimer may occur, this has been documented in other systems such as the change in abundance of Fos family members with induction of 3T3 cells by serum where Fos is replaced by Fra (Vosatka *et al*, 1989). The requirement for

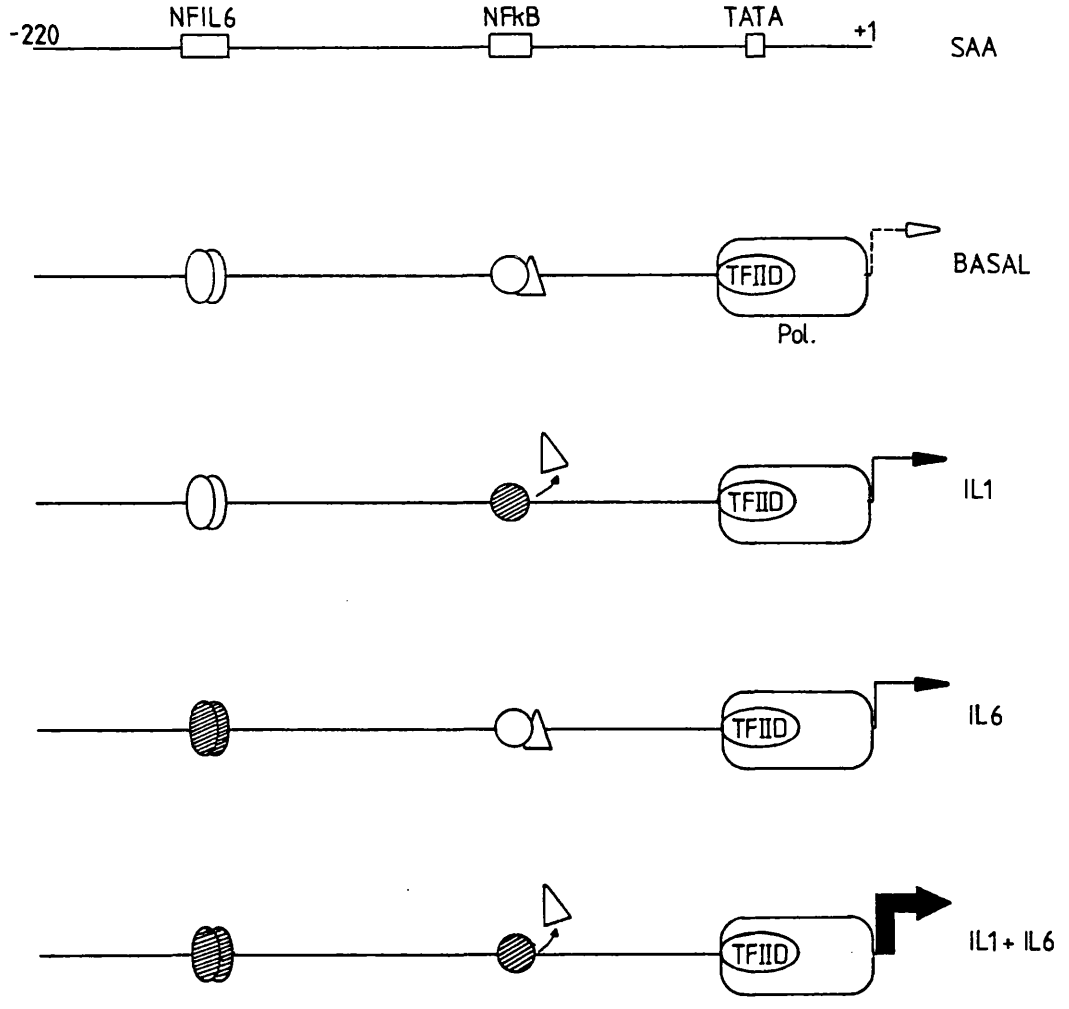


Figure 6.2 A model for transcription factor interactions during acute phase control of SAA gene transcription.

A model is shown for proteins interacting with the SAA promoter at the NFκB and NF-IL6 regions under different conditions of stimulation. The NFκB-like factor is represented as a circle, NF-IL6-like factors as dimers and complex II proteins as a triangle. Constitutively present factors are shown *unshaded*, and cytokine induced factors *shaded* to indicate modification and/or a quantitative increase of the factor. The induced factors may interact cooperatively with the RNA polymerase II complex shown schematically on the right to activate transcription (*arrow*). For further details of the model refer to text.

NFκB is demonstrated by the reduction in the induction ratio when this site is mutated. The contribution from the IL6RE is indicated by reduced induction of *SAA2CAT*100. That other (complex II) proteins are also induced in the NFκB region is supported by the finding that *SAA2CAT*100 transcription consistently falls below its own constitutive level 16 hours after IL6 treatment (figure 5.8), presumably due to an increased level of these proteins competing for binding of the basal level of NFκB necessary for transcription.
iv) IL1 and IL6: Induction of both NFκB and C/EBP-like proteins leads to synergistic activation of transcription, possibly through the induced proteins simultaneously interacting with the transcriptional machinery. The importance of the individual proteins concerned in the above series of events will be confirmed by further time course studies using mutants at the IL6RE and complex II binding site. This model is summarised in figure 6.2.

A number of other genes, particularly those for cytokines have both NF-IL6 and NFκB binding sites in their promoters. These include IL6 (Isshiki *et al*, 1990), TNFα (Hensel *et al*, 1989) and the gene for the T cell and neutrophil chemotactic factor IL8 (Mukaida *et al*, 1990). In the IL6 gene both elements are probably required for full induction by other cytokines as individual reports have documented the requirement of NFκB (Shimizu *et al*, 1990; Libermann and Baltimore, 1990) and NF-IL6 (Isshiki *et al*, 1990). Whereas the involvement of the NFκB sites in the TNF promoter has been demonstrated (Collart *et al*, 1990) the regulatory capacity of the putative NF-IL6 site between -196 and -183 has not. In the IL8 gene the NF-IL6 and NFκB sites are directly apposed between -94 and -71, both are essential for induction by IL1, TNF and PMA but did not function individually to activate an heterologous promoter (Mukaida *et al*, 1990).

These studies demonstrate the fundamental importance of NFκB-like and C/EBP-like factors in regulation of the genes in the acute phase and inflammatory response. Further work will characterize the molecular interactions which occur in producing specificity of gene regulation under conditions where multiple cytokines are active.

CHAPTER 7

Conclusions and Perspectives.

7.1 Physical Structure of Human SAA Genes.

In this work the human SAA gene family was demonstrated to comprise 4 discrete loci with allelic variants identified for *SAA1* and *SAA2*. Three loci were cloned from genomic libraries, *SAA1*β, *SAA2*α/*SAA2*β and *SAA4*. While *SAA2* and *SAA4* were 10kb apart, linkage was not established with the other genes via cloning. These studies can be immediately extended by the sequencing of the *SAA4* clone to predict a gene product and determine the evolutionary relatedness to the other three genes. Although *SAA3* has been cloned in two labs (Sack and Talbot, 1989; Kluve-Beckerman *et al*, 1991a) we have not obtained a clone to date. Using oligonucleotide primers based on the published sequence of *SAA3* a fragment spanning exons 3 to 4, amplified by polymerase chain reaction (PCR) will be used as a specific probe to screen genomic libraries. This will allow preferential isolation of *SAA3* over *SAA1*/*SAA2*. The characterization of *SAA3* is important with regards to the recent data of Kluve-Beckerman *et al* who found an *SAA3* genomic clone HDg1.1 with a T residue insertion between codons 31 and 32 in exon 3. This would cause a frame-shift mutation and result in a premature translation termination product of only 42 amino acids (codon 43 would be TGA, Kluve-Beckerman *et al*, 1991a). The extra T residue was also present in a PCR amplified fragment of *SAA3* from 4 other individuals and thus contrasts with the data of Sack and Talbot (1989). Three other nucleotide differences were identified in exon 3 prior to codon 31 and 4 in the remainder of the exon whereas exons 2 and 4 were identical. It will be interesting to determine which alleles are present in our population.

Physical mapping of the SAA locus will be completed by screening of genomic libraries in yeast artificial chromosome (YAC) vectors. A YAC library (gift of R. Anend, ICI) will be screened by PCR of pooled clones with SAA-specific oligonucleotides. "Sub-pools" of positives are rescreened until individual clones are isolated. YAC vectors, originally developed by Olson and colleagues carry inserts of several hundred kb (Burke *et al*, 1987), and would allow isolation of the entire SAA locus on one, or a few contiguous clones. Another aim for the structural studies would be to further localize the SAA gene locus on chromosome 11p. This can be done using a combination of physical: somatic cell hybrids (Wadey *et al*, 1990) and pulsed field analysis (see chapter 4); and genetic methods using the RFLPs identified as markers. Finally patient populations may be screened for linkage between SAA gene polymorphisms and the occurrence of AA-type amyloidosis either secondary to inflammatory disease (*e.g.* juvenile chronic arthritis) or inherited diseases such as FMF (although this was not established with PstI and TaqI polymorphisms; Shohat *et al*, 1990).

7.2 Comparative Studies of Human SAA Gene Expression.

To date extensive comparative studies of the expression of individual SAA genes have only been carried out in the mouse. As described in section 1.2, generally *SAA1* and *SAA2* maintain a similar pattern of expression in response to different stimuli whereas *SAA3* expression is disparate. In transient transfection studies the conserved regions of the *SAA1* and *SAA2* promoters (up to -440bp) were found to respond in a comparable fashion to IL1 and IL6 (section 5.2.2). However as discussed (section 6.1) SAA1 is the predominant isotype found in human acute phase plasma. Thus further systematic studies using deletion constructs will be necessary to establish the role of the sequences 5' of -440bp in regulating transcription. Whereas *SAA2* has a second NFκB site (-635 to -626) which modulates transcription (Edbrooke *et al*, 1991) and an adjacent putative C/EBP site, this region is degenerate in *SAA1*. However the so-called B region of *SAA1* (see figure 5.1) contains a possible NFκB site 5'-GGGCTTTCT-3' on the coding strand and a putative C/EBP site 5'-TAATTCCGAATTG-3' a further 16bp upstream (figure 6.1). Thus the relative orientations of these sites is the same as *SAA2* and may be of functional significance.

Further levels of gene control may also be studied with cell lines (*e.g.* HepG2) permanently transfected with clones containing the individual SAA genes including mRNA stability and secretion rates. Alternatively cell lines in which the endogenous SAA genes are expressed such as the human hepatoma line HuH7 could be utilized and RNA products distinguished with gene-specific probes.

The availability of human SAA clones will enable functional issues to be addressed. Work is currently underway in this laboratory to characterize the SAA1α protein expressed from pA1 under the CMV promoter in CHO cells. The lipid binding characteristics and possible degradation to AA-like forms by serum proteases will be examined in addition to structural analysis by X-ray crystallography. A starting point for functional studies maybe to determine the effect of purified SAA on different cell types *i.e.* do cellular or secreted proteins have their production altered by SAA. This can be pursued at a crude level or alternatively specific genes involved in inflammation (*e.g.* cytokine) or inflammatory disease (*e.g.* collagenase which is induced by rabbit SAA, Mitchell *et al*, 1991) could be studied. The observation that SAA activates collagenase in fibroblasts (Mitchell *et al*, 1991) implies the existence of a cellular receptor for SAA. Using recombinant SAA, cells expressing a receptor could be identified and would be candidates for characterizing such a molecule using established techniques (*e.g.* as used for the IL1R, Dower *et al*, 1985; Sims *et al*, 1988). Potential second and third messengers modulated by SAA could also be investigated.

7.3 Transcription Factors and Transcriptional Regulation.

NFκB- and C/EBP-like proteins bind to the SAA promoter and are important for regulating transcription. Experiments such as those described in chapter 6 would further

define the transcriptional response to cytokines. The most immediate of these is to mutate the putative IL6RE within the context of the SAA promoter and analyze the effect of this mutation in transfection studies.

Currently an area of intense investigation in a number of laboratories is centred on defining the mechanisms of synergistic activation of transcription by combinations of *trans*-acting factors. A major question arising from the current study is by what mechanism do IL1 and IL6 regulated factors synergistically activate transcription? For example do these factors bind in a cooperative manner to their sites in the SAA promoter? The possibilty of cooperative binding of NFκB and C/EBP factors to DNA can be tested for by footprinting experiments. For example, given the same concentration of protein in an extract binding to the SAA NF-IL6 site, determine whether the footprint is enhanced in the presence of NFκB binding to the same fragment at its recognition sequence (*i.e.* carry out the same experiment with wild-type and mutated NFκB sites). Similar experiments by Li *et al* (1991) demonstrated increased binding of the E2 protein of bovine papilloma virus with increasing amounts of Sp1 protein to a DNA fragment containing recognition sites for both proteins. It maybe possible to distinguish alterations in the subunit structure of constitutively bound and induced factors using antibodies raised against different members of the C/EBP and NFκB families. Anti-C/EBP antibodies were used to show that some of the proteins binding to the angiotensinogen APRE belonged to this family (Brasier *et al*, 1990). Potential modifications of transcription factors include altered phosphorylation status; variation of phosphorylation conditions may differentially enhance or diminish binding in untreated and cytokine treated nuclear extracts. For example, the presence or absence of nucleoside triphosphates altered the binding of IFN-inducible factors (Roy and Lebleu, 1990) whereas phosphatase treatment abrogated the enhanced binding of bacterially expressed SRF by HeLa cell extracts (Manak *et al*, 1990). Alternatively protein-protein interactions (inter- or intra-chain) maybe altered following cytokine treatment. Simple mobility shift assays can demonstrate formation of different molecular weight complexes as found for human oestrogen receptor-DNA complexes following various treatments (Brown and Sharp, 1990).

The studies on transcription factor interactions will be limited by the use of crude nuclear extracts. Purified factors may be obtained by fractionation of extracts and use of affinity columns carrying the binding sites of interest. Sequence obtained from the purified proteins can be used to design primers for screening of human liver cDNA libraries. This technique has been used to successfully purify and clone a number of factors including the p50 subunit of NFκB (Kieran *et al*, 1990; Ghosh *et al*, 1990). Other possibilities include screening an expression library with the DNA recognition sequence (*e.g.* Fan and Maniatis, 1990) although this does not allow for the isolation of heterodimeric factors, or low stringency hybridization with an available clone known to belong to the same transcription factor family (*e.g.* Rustgi *et al*, 1990). The proteins

isolated can then be used to carry out experiments such as those described above. In addition it will be possible to test for direct protein-protein interaction. For example does anti-C/EBP antibody preciptate a complex of NFκB bound to its motif in the presence of C/EBP? Using electron microscopy Li *et al* (1991) showed direct interaction between E2 and Sp1 bound to their recognition sequences separated by 780bp of intervening DNA which was looped out. Protein interactions may also be examined by altering the spacing between the two binding sites and studying the effect on transcriptional induction to determine whether stereospecific alignment of proteins on the DNA is essential. Gutman and Wasylyk (1990) found the spacing between AP-1 and PEA3 motifs to be important in modulating synergistic activation of the collagenase promoter suggesting the importance of stereospecific alignment. Similar studies by Schatz and Chatton (1990) revealed association between a 10bp periodicity in the spacing of two binding sites and the ability to activate transcription over spacings below 125bp.

The availabilty of cloned proteins will also allow experiments such as those carried out by Ptashne and colleagues to test for cooperative binding. If the proteins are present at high enough concentrations to saturate their binding sites on the DNA but the same synergistic transcriptional response is obtained as at non-saturating levels then cooperative binding of factors is not making a contribution to synergism (Carey *et al*, 1990; Lin *et al*, 1990).

Another area of interest would be to define the cellular targets of transcription factors involved in SAA transcription, particularly if C/EBP and NFκB proteins synergistically induce transcription but cooperative binding and/or protein-protein interactions do not occur. The evidence for interaction of transcription factors with general initiation factors and adaptor molecules was discussed in section 1.5. Progressive fractionation of a cellular extract can be used to purify fractions necessary to reconstitute full *in vitro* transcription by the *trans*-activator of interest. This procedure allowed Flanagan *et al* (1991) to purify a mediator (adaptor) which could relieve squelching by GAL4-VP16 and was required by both this factor and GCN4 for maximal activity. It would be possible to determine whether IL6- and IL1-regulated factors required the same fractions and thus interact with similar or different targets at the SAA promoter. Finally although the experiments described will be important in defining fundamental processes occurring at transcription, their relevance *in vivo* must be demonstrated where higher orders of control such as chromatin structure are manifest. Thus techniques such as *in vivo* footprinting (Mirkovitch and Darnell, 1991) maybe employed to determine the occupancy of recognition sequences during the APR.

In conclusion this study resulted in the delineation of the human SAA gene family and the demonstration of transcriptional regulation by cytokines of two of the genes. Future experiments described above are among several possibilities which maybe followed to obtain a more complete understanding of the molecular mechanisms involved in regulation of the acute phase response.

REFERENCES.

Abraham LJ, Bradshaw AD, Shiels BR, Northemann W, Hudson G, Fey GH. (1990) Hepatic transcription of the acute phase α_1-inhibitor III gene is controlled by a novel combination of *cis*-acting regulatory elements. *Mol. Cell. Biol.* **10**, 3483-3491.

Abraham RT, Ho SN, Barna TJ, McKean DJ. (1987) Transmembrane signalling during IL1-dependent T cell activation. *J. Biol. Chem.* **262**, 2719-2728.

Aderka D, Le J, Vilcek J. (1989) IL6 inhibits LPS-induced TNF production in cultured human monocytes, U937 cells, and in mice. *J. Immunol.* **143**, 3517-3523.

Adrian GS, Korinek BW, Bowman BH, Yang F. (1986) The human transferrin gene: 5' region contains conserved sequences which match the control elements regulated by heavy metals, glucocorticoids and acute phase reaction. *Gene* **49**, 167-175.

Aggarwal BB, Eessalu TE, Hass PE. (1985) Characterization of receptors for human TNF and their regulation by gamma-IFN. *Nature* **318**, 665-667.

Akahoshi T, Oppenheim JJ, Matsushima K. (1988) Induction of high affinity IL1-receptor on human peripheral blood lymphocytes by glucocorticoid hormones. *J. Exp. Med.* **167**, 924-936.

Akerblom IE, Slater EP, Beato M, Baxter JD, Mellon PL. (1988) Negative regulation by glucocorticoids through interference with a cAMP responsive enhancer. *Science* **240**, 350-353.

Akira S, Hirano T, Taga T, Kishimoto T. (1990) Biology of multifunctional cytokines: IL6 and related molecules (IL1 and TNF) *FASEB J.* **4**, 2860-2867.

Akira S, Isshiki H, Sugita T, Tanabe O, Kinoshita S, Nishio Y, Nakajima T, Hirano T, Kishimoto T. (1990) A nuclear factor for IL6 gene expression (NF-IL6) is a member of a C/EBP gene family. *EMBO J.* **9**, 1897-1906.

Albrightson CR, Baenziger NL, Needleman P, (1985) Exaggerated human vascular cell PG biosynthesis mediated by monocytes: role of monokines and IL1. *J. Immunol.* **135**, 1872-1877.

Aldo-Benson MA, Benson MD. (1982) SAA suppression of immune response *in vitro*: evidence for an effect on T cell macrophage interaction. *J. Immunol.* **128**, 2390-2392.

Aldred AR, Grimes A, Schreiber G, Mercer JF. (1987) Rat ceruloplasmin. Molecular cloning and gene expression in liver, choroid plexus, yolk sac, placenta and testis. *J. Biol. Chem.* **262**, 2875-2878.

Alexander F, Young PR, Tilghman SM. (1984) Evolution of the albumin: α-fetoprotein ancestral gene from the amplification of a 27bp nucleotide sequence. *J. Mol. Biol.* **173**, 159-176.

Allen G, Fantes KH. (1980) A family of structural genes for human lymphoblastoid (leukocyte type) interferon. *Nature* **287**, 408-411.

Anderson H, McCarty M. (1951) The occurrence in the rabbit of an APP analogous to human CRP. *J. exp. Med.* **93**, 25-36.

Andus T, Geiger T, Hirano T, Kishimoto T, Heinrich PC. (1988a) Action of recombinant human interleukin 6, interleukin 1ß and tumour necrosis factor α on the mRNA induction of acute-phase proteins. *Eur. J. Immunol.* **18**, 739-746.

Andus T, Geiger T, Hirano T, Kishimoto T, Tran-Thi T-A, Decker K, Heinrich PC. (1988b) Regulation of the synthesis and secretion of major rat acute-phase proteins by recombinant human interleukin-6 (BSF-2/IL-6) in hepatocyte primary cultures. *Eur. J. Biochem.* **173**, 287-293.

Angel P, Hattori K, Smeal T, Karin M. (1988) The *jun* proto-oncogene is positively autoregulated by its product Jun/AP-1. *Cell* **55**, 875-885.

Ann DK, Smith MK, Carlson DM. (1988) Molecular evolution of the mouse proline-rich protein gene family. *J. Biol. Chem.* **263**, 10887-10893.

Arden B, Klotz JL, Siu G, Hood LE. (1985) Diversity and structure of genes of the α family of mouse T-cell antigen receptor. *Nature* **316**, 783-787.

Antiquera F, Boyes J, Bird A. (1990) High levels of *de novo* methylation and altered chromatin structure at CpG islands in cell lines. *Cell* **62**, 503-514.

Arcone R, Gualandi G, Ciliberto G. (1988) Identification of sequences responsible for acute-phase induction of human C-reactive protein. *Nucl. Acids Research* **16**, 3195-3207.

Assoian RK, Sporn MB. (1986) Type beta TGF in human platelets: release during platelet degranulation and action on smooth muscle cells. *J. Cell Biol.* **102**, 1217-1223.

Atkins E. (1960) Pathogenesis of fever. *Physiol. Rev.* **40**, 580-646.

Auron PE, Warner SJC, Wabb AC, Cannon JG, Bernheim HA, McAdam KPWJ, Rosenwasser LJ, LoPreste G, Mucci SF, Dinarello, CA (1987) Studies on the molecular nature of human IL1. *J. Immunol.* **138**, 1447-1456.

Auron PE, Webb AC, Rosenwasser LJ, Mucci SF, Rich A, Wolff SM, Dinarello CA. (1984) Nucleotide sequence of human monocyte IL1 precursor cDNA. *Proc. Natl. Acad. Sci. USA* **81**, 7907-7911.

Baeuerle PA, Baltimore D. (1988a) Activation of DNA binding activity in an apparently cytoplasmic precursor of the NFκB transcription factor. *Cell* **53**, 211-217.

Baeuerle PA, Baltimore D. (1988b) IκB: a specific inhibitor of the NFκB transcription factor. *Science* **242**, 540-546.

Baeuerle PA, Baltimore D. (1989) A 65kD subunit of active NFκB is required for inhibition of NFκB by NFκB. *Genes Dev.* **3**, 1689-1698.

Baglioni C, McCandless S, Tavernier J, Fiers W.(1985) Binding of human TNF to high affinity receptors on HeLa and lymphoblastoid cells sensitive to growth inhibition. *J. Biol. Chem.* **260**, 13395-13397.

Ballard DW, Walker WH, Doerre S, Sista P, Molitor JA, Dixon EP, Peffer NJ, Hannink M, Greene WC. (1990) The v-*rel* oncogene encodes a κB enhancer binding protein that inhibits NFκB function. *Cell* **63**, 803-814.

Ballou SP, Buniel J, Macintyre SS. (1989) Specific binding of human CRP to monocytes *in vitro*. *J. Immunol.* **142**, 2708-2713.

Baltimore D. (1981) Gene conversion: some implications for Ig genes. *Cell* **24**, 592-594.

Baltz ML, Rowe IF, Caspi D, Turnell WG, Pepys MB. (1987) Acute-phase high-density lipoprotein in the rat does not contain serun amyloid A protein . *Biochem. J.* **242**, 301-303.

Baumann H. (1989) Hepatic acute phase reaction *in vivo* and *in vitro*. *In Vitro Cell. Dev. Biol.* **25**, 115-126.

Baumann H, Hill RE, Sauder DN, Jahreis GP. (1986) Regulation of major acute-phase plasma proteins by hepaotcyte-stimulating factors of human squamous carcinoma cells. *J. Cell. Biol.* **102**, 370-383.

Baumann H, Jahreis GP, Sauder DN, Koj A. (1984) Human keratinocytes and monocytes release factors which regulate the synthesis of major acute phase plasma proteins in hepatic cells from man, rat, and mouse. *J. Biol. Chem.* **259**, 7331-7342.

Baumann H, Maquat LE. (1986) Localization of DNA sequences involved in dexamethasone-dependent expression of the rat α_1AGP gene. *Mol. Cell. Biol.* **6**, 2551-2561.

Baumann H, Morella KK, Jahreis GP, Marinkovic S. (1990) Distinct regulation of the IL1 and IL6 response elements of the rat haptoglobin gene in rat and human hepatoma cells. *Mol. Cell. Biol.* **10**, 5967-5976.

Baumann H, Muller-Eberhard U. (1987) Synthesis of haptoglobin and cysteine proteinase inhibitoris coordinately regulated by HSF-II and IFNß$_2$ in rat hepatoma cells. *Biochem. Biophys. Res. Comm.* **146**, 1218-1226.

Baumann H, Onorato V, Gauldie J, Jahreis GP. (1987a) Distinct sets of acute phase plasma proteins are stimulated by separate human hepatocyte-stimulating factors and monokines in rat hepatoma cells. *J. Biol. Chem.* **262**, 9756-9768.

Baumann H, Richards C, Gauldie J. (1987b) Interaction among hepatocyte-stimulating factors, interleukin 1, and glucocorticoids for regulation of acute phase plasma proteins in human hepatoma (HepG2) cells. *J. Biol. Chem.* 262, 4122-4128.

Baumann H, Won K-A, Jahreis GP. (1989) HSFIII and IL6 are structurally and immunologically distinct but regulate the production of the same acute phase plasma proteins. *J. Biol. Chem.* **264**, 8046-8051.

Baumann H, Wong GG. (1989) Hepatocyte-stimulating factor III shares structural and functional identity with leukaemia-inhibitory factor. *J. Immunol.* **143**, 1163-1167.

Baumruker T, Sturm RA, Herr W. (1988) OBP100 binds remarkably degenerate octamer motifs through specific interactions with flanking sequences. *Genes Dev.* **2**, 1400-1403.

Bausserman LL, Herbert PN, McAdam KPWJ. (1980) Heterogeneity of human serum amyloid A proteins. *J. Exp. Med.* **152**, 641-656.

Bausserman LL, Saritelli AL, Herbert PN, McAdam KPWJ, Shulman RS. (1982) NH_2-terminal analysis of four of the polymorphic forms of human serum amyloid A proteins. *Biochim. Biophys. Acta* **704**, 556-559.

Bausserman LL, Saritelli AL, Van Zuiden P, Gollaher CJ, Herbert PN. (1987) Degradation of serum amyloid A by isolated perfused rat liver. *J. Biol. Chem.* **262**, 1583-1589.

Bazan JF. (1990) Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl. Acad. Sci. USA* **87**, 6934-6938.

Benbrook DM, Jones NC. (1990) Heterodimer formation between CREB and JUN proteins. *Oncogene* **5**, 295-302.

Benditt EP, Eriksen N (1971) Chemical classes of amyloid substance. *Am. J. Pathol.* **65**, 231-252.

Benditt EP, Eriksen N. (1977) Amyloid protein SAA is associated with HDL from human serum. *Proc. Natl. Acad. Sci. USA* **74**, 4025-4028.

Benditt EP, Meek RL. (1989) Expression of the third member of the SAA gene family in mouse adipocytes. *J. Exp. Med.* **169**, 1841-1846.

Benjamin D, Wormsley S, Dower SK. (1990) Heterogeneity in IL1-receptors expressed on human B cell lines. *J. Biol. Chem.* **265**, 9943-9951.

Bensimon C, Wakasugi H, Wakasugi N, Jitsukawa S, Tursz T. (1989) A monoclonal antibody recognizing 68- to 75-kilodalton protein(s) associated with the human IL-1 receptor. *J. Immunol.* **142**, 2290-2298.

Benson MD, Aldo-Benson MA. (1979) Effect of purified protein SAA on immune responses *in vitro*, mechanisms of suppression. *J. Immunol.* **122**, 2077-2082.

Benson MD, Aldo-Benson MA, Shirahama T, Borel Y, Cohen AS. (1975) Suppression of an *in vivo* antibody response by a serum factor (SAA) in experimentally induced amyloidosis. *J. Exp. Med.* **142**, 236-241.

Benson MD, DiBartola SP, Dwulet FE. (1989) A unique insertion in the primary structure of bovine amyloid AA protein. *J. Lab. clin. Med.* **113**, 67-72.

Benson MD, Skinner M, Lian J, Cohen AS. (1975) "A" protein of amyloidosis. Isolation of a cross-reacting component from serum by affinity chromatography. *Arth. Rheum.* **18**, 315-322.

Benton MD, Davis RW. (1977) Screening λgt recombinant clones by hybridization to single plaques *in situ*. *Science* **196**, 180-182.

Bereta J, Kurdowska A, Koj A, Hirano T, Kishimoto T, Content J, Fiers W, VanDamme J, Gauldie J. (1989) Different preparations of natural and recombinant human IL-6 (IFNß$_2$, BSF-2) similarly stimulate acute phase protein synthesis and uptake of α-aminoisobutyric acid by cultured rat hepatocytes. *Int. J. Biochem.* **21**, 361-366.

Bereta J, Szuba K, Fiers W, Gauldie J, Koj A. (1990) Transforming growth factor-ß and epidermal growth factor modulate basal and interleukin-6-induced uptake and acute phase protein synthesis in cultured rat hepatocytes. *FEBS Lett.*, **266**, 48-50.

Berger S, Cress WD, Cress A, Triezenberg SJ, Guarente L. (1990) Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. *Cell* **61**, 1199-1208.

Berk AJ. (1989) Regulation of eukaryotic transcription factors by post-translational modification. *Biochim. Biophys. Acta* **1009**, 103-109.

Berkenbosch F, van Oers J, del Rey A, Tilders F, Besedovsky H. (1987) Corticotropin releasing factor neurons in the rat activated by IL1. *Science* **238**, 524-528.

Bernton EW, Beach JE, Holaday JW, Smallbridge RC, Fein HG. (1987) Release of multiple hormones by direct action of interleukin-1 on pituitary cells. *Science* **238**, 519-521.

Besedovsky H, del Rey A, Sorkei E, Dinarello CA. (1986) Immunoregulatory feedback between IL1 and glucocorticoid hormones. *Science* **233**, 652-654.

Beutler B, Cerami A. (1988a) The history, properties, and biological effects of cachectin. *Biochemistry* **27**, 7575-7582.

Beutler B, Cerami A. (1988b) Tumour necrosis, cachexia, shock, and inflammation: a common mediator. *Annu. Rev. Biochem.* **57**, 505-518.

Birch HE, Schreiber G. (1986) Transcriptional regulation of plasma protein synthesis during inflammation. *J. Biol. Chem.* **261**, 8077-8080.

Bird AP. (1980) DNA methylation and the frequency of CpG in animal DNA. *Nucl. Acids Research* **11**, 1499-1504.

Bird AP, Taggart MH, Nicholls RD, Higgs DR. (1987) Non-methylated CpG-rich islands at the human α-globin locus: implications for evolution of the α-globin pseudogene. *EMBO J.* **6**, 999-1004.

Bird TA, Gearing GH, Saklatvala J. (1987) Murine IL1-R: differences in binding properties between fibroblastic and thymoma cells and evidence for a 2-chain receptor model. *FEBS Lett.* **225**, 21-26.

Bird TA, Gearing AJH, Saklatvala J. (1988) Murine IL1-R. Direct identification by ligand blotting and purification to homogeneity of an IL1-binding glycoprotein. *J. Biol. Chem.* **263**, 12063-12069.

Bird TA, Saklatvala J. (1986) Identification of a common class of high affinity receptors for both types of porcine IL1 on connective tissue cells. *Nature* **324**, 263-266.

Bird TA, Saklatvala J. (1989) IL1 and TNF transmodulate EGF receptors by a protein kinase C independent mechanism. *J. Immunol.* **142**, 126-133.

Blackwood EM, Eisenmann RN. (1991) Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. *Science* **251**, 1211-1217.

Bohnlein E, Lowenthal JW, Siekevitz M, Ballard DW, Franza BR, Greene WC. (1988) The same inducible nuclear protein regulates mitogen activation of both the IL2Rα gene and type I HIV. *Cell* **53**, 827-836.

Bohmann D, Bos TJ, Admon A, Nishimura T, Vogt PK, Tjian R. (1987) Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. *Science* **238**, 1386-1392.

Bomsztyk K, Sims JE, Stanton TH, Slack J, McMahan CJ, Valentine MA, Dower SK. (1989) Evidence for different IL1 receptors on murine B- and T-cell lines. *Proc. Natl. Acad. Sci. USA* **86**, 8034-8038.

Bomsztyk K, Toivola B, Emery DW, Rooney JW, Dower SK, Rachie NA, Hopkins Sibley C. (1990) Role of cAMP in IL1-induced k light chain gene expression in murine B cell line. *J. Biol. Chem.* **265**, 9413-9417.

Botstein D, White RL, Skolnick M, Davis RW. (1980) Construction of a genetic linkage map using RFLPs. *Am. J. Hum. Gen.* **32**, 314-331.

Bours V, Villalobos J, Burd PR, Kelly K, Siebenlist U. (1990) Cloning of a mitogen-inducible gene encoding a κB DNA-binding protein with homology to the *rel* oncogene and to cell cycle motifs. *Nature* **348**, 76-80.

Braciak TA, Northemann W, Hudson GO, Shiels BR, Gehring MR, Fey GH. (1988) Sequence and acute phase regulation of rat α_1-inhibitor III messenger RNA. *J. Biol. Chem.* **263**, 3999-4012.

Brasier AR, Ron D, Tate JE, Habener JF. (1990) A family of constitutive C/EBP-like DNA binding proteins attenuate the IL1 induced, NFκB mediated *trans*-activation of the angiotensinogen gene APRE. *EMBO J.* **9**, 3933-3944.

Brenner DA, O'Hara M, Angel P, Chojkier M, Karin M. (1989) Prolonged activation of *jun* and collagenase genes by TNFα. *Nature* **337**, 661-663.

Brent R, Ptashne M. (1985) A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. *Cell* **43**, 729-736.

Brinckerhoff CE, Mitchell TI, Karmilowicz MJ, Kluve-Beckerman B, Benson MD. (1989) Autocrine induction of collagenase by serum amyloid A-like and β_2-microglobulin-like proteins. *Science* **243**, 655-657.

Brissette L, Young I, Narindrasorasak S, Kisilevsky R, Deeley R. (1989) Differential induction of the serum amyloid A gene family in response to an inflammatory agent and to amyloid-enhancing factor. *J. Biol. Chem.* **264**, 19327-19332.

Britten RJ, Baron WF, Stout DB, Davidson EH. (1988) Sources and evolution of the human *Alu* repeated sequences. *Proc. Natl. Acad. Sci. USA* **85**, 4770-4774.

Brown M, Sharp PA. (1990) Human estrogen receptor forms multiple protein-DNA complexes. *J. Biol. Chem.* **265**, 11238-11243.

Bruggemeier U, Rogge L, Winnacker E-L, Beato M. (1990) NFI acts as a transcription factor on the MMTV promoter but competes with steroid hormone receptor for DNA binding. *EMBO J.* **9**, 2233-2239.

Buchta R, Pontet M, Fridkin M. (1987) Binding of CRP protein to human neutrophils. *FEBS Lett.* **211**, 165-168.

Bull P, Morley KL, Hoekstra MF, Hunter T, Verma IM. (1990) The mouse c-*rel* protein has an N-terminal regulatory domain and a C-terminal transcriptional transactivation domain. *Mol. Cell. Biol.* **10**, 5473-5485.

Burke DT, Carle GF, Olson M. (1987) Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. *Science* **236**, 806-812.

Calame KL. (1989) Immunoglobulin gene transcription: molecular mechanisms. *Trends Genet.* **5**, 395-399.

Cao SX, Gutman PD, Dave HPG, Schecter AN. (1989) Identification of a transcriptional silencer in the 5' flanking region of the human ε globin gene. *Proc. Natl. Acad. Sci. USA* **86**, 5306-5309.

Carey M, Lin Y-S, Green MR, Ptashne, M. (1990) A mechanism for synergistic activation of a mammalian gene by GAL4 derivatives. *Nature* **345**, 361-364.

Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. (1975) An endotoxin-induced serum factor that causes necrosis of tumours. *Proc. Natl. Acad. Sci. USA* **72**, 3666-3670.

Castell JV, Gomez-Lechon MJ, David M, Hirano T, Kishimoto T, Heinrich PC. (1988) Recombinant human interleukin-6 regulates the synthesis of acute phase proteins in human hepatocytes. *FEBS Lett.* **232**, 347-350.

Castell JV, Gomez-Lechon MJ, David M, Andus T, Geiger T, Trullenque R, Fabra R, Heinrich PC. (1989) Interleukin-6 is the major regulator of acute phase protein synthesis in adult human hepatocytes. *FEBS Lett.* **242**, 237-239.

Cedar H. (1988) DNA methylation and gene activity. *Cell* **53**, 3-4.

Cereghini S, Raymondjean M, Garcia Carranca A, Herbomel P, Yaniv M. (1987) Factors involved in control of tissue-specific expression of albumin gene. *Cell* **50**, 627-638.

Chang C-J, Chen TT, Lei H-Y, Chen D-S, Lee S-C. (1990) Molecular cloning of a transcription factor, AGP/EBP, that belongs to members of the C/EBP family. *Mol. Cell. Biol.* **10**, 6642-6653.

Chedid M, Mizel SB. (1990) Involvement of cyclic AMP-dependent protein kinases in the signal transduction pathway for interleukin 1. *Mol. Cell. Biol.* **10**, 3824-3827.

Chedid M, Shirakawa F, Naylor P, Mizel SB. (1989) Signal transduction pathway for IL1. Involvement of a pertussis toxin-sensitive GTP-binding protein in the activation of adenylate cyclase. *J. Immunol.* **142**, 4301-4306.

Chen L, Suzuki Y, Wheelock EF. (1987) IFNγ synergizes with TNF and with IL1 and requires the presence of both monokines to induce antitumour cytotoxic activity in macrophages. *J. Immunol.* **139**, 4096-4101.

Cheresh DA, Haynes DH, Distasio JA. (1984) Interaction of an APR, α_1AGP (orosomucoid) with the lymphoid cell surface: a model for non-specific and immune suppression. *Immunology* **51**, 541-548.

Cherry JR, Johnson TR, Dollard C, Shuster JR, Denis CL. (1989) cAMP-dependent protein kinase phosphorylates and inactivates the yeast transcriptional activator ADR1. *Cell* **56**, 409-419.

Cheung DL, Hart PH, Vitti GF, Whitty GA, Hamilton JA. (1990) Contrasting effects of IFNγ and IL4 on the IL6 activity of stimulated human monocytes. *Immunol.* **71**, 70-75.

Chin J, Rupp E, Cameron PM, MacNaul KL, Lotke PA, Tocci MJ, Schmidt JA, Bayne EK. (1988) Identification of a high affinity receptor for IL1α and IL1ß on cultured human rheumatoid synovial cells. *J. Clin. Invest.* **82**, 420-426.

Chizzonite R, Truitt T, Kilian PL, Stern AS, Nunes P, Parker KP, Kaffka KL, Chua AO, Lugg DK, Gubler U. (1989) Two high-affinity IL1 receptors represent separate gene products. *Proc. Natl. Acad. Sci. USA* **86**, 8029-8033.

Christy RJ, Yang VW, Ntambi JM, Geiman DE, Landschulz WH, Friedman AD, Nakabeppu Y, Kelly TJ, Lane MD. (1989) Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte specific genes. *Genes Dev.* **3**, 1323-1325.

Clark BD, Collins KL, Gandy MS, Webb AC, Auron PE. (1986) Genomic sequence for human IL1ß: possible evolution from a reverse transcribed proIL1α gene. *Nucl. Acids Research* **14**, 3167-3179.

Clark EA, Shu G. (1990) Association between IL6 and CD40 signalling. *J. Immunol.* **145**, 1400-1406.

Coe JE. (1977) A sex limited serum protein of Syrian hamsters: definition of female protein and regualtion by testosterone. *Proc. Natl. Acad. Sci. USA* **74**, 730-

Coe JE, Ross MJ. (1983) Hamster female protein: a divergent APP in male and female Syrian hamsters. *J.Exp.Med.* **157**, 1421-

Coetzee GA, Strachan AF, van der Westhuyzen DR, Hopper HC, Jeenah MS, de Beer FC. (1986) Serum amyloid A-containing human high density lipoprotein 3: density, size, and apolipoprotein composition. *J. Biol. Chem.* **261**, 9644-9651.

Cole T, Inglis AS, Roxburgh CM, Howlett GJ, Schreiber G. (1985) Major acute phase alpha$_1$-protein of the rat is homologous to bovine kininogen and contains the sequence for bradykinin: its synthesis is regulated at the mRNA level. *FEBS Lett.* **182**, 57-61.

Collart MA, Baeuerle P, Vassali P. (1990) Regulation of TNFα transcription in macrophages: involvement of 4 κB motifs and of constitutive and inducible forms of NFκB. *Mol. Cell Biol.* **10**, 1498-1506.

Colotta F, Lampugnani MG, Polentarutti N, Dejana E, Mantovani A. (1988) IL1 induces c-*fos* protooncogene expression in cultured human endothelial cells. *Biochem Biophys. Res. Comm.* **152**, 1104-1110.

Conner J, Vallet-Collom I, Daveau M, Delers F, Hiron M, Lebreton J-P, Guillouzo A. (1990) Acute-phase-response induction in rat hepatocytes co-cultured with rat liver epithelial cells. *Biochem. J.* **266**, 683-688.

Cook PR. (1989) The nucleoskeleton and the topology of transcription. *Eur. J. Biochem.* **185**, 487-501.

Costa PP, Figueira AS, Bravo FR. (1978) Amyloid fibril protein related to prealbumin in familial amyloidotic polyneuropathy. *Proc. Natl. Acad. Sci. USA* **75**, 4499-4503.

Costa RH, Grayson DR, Darnell JE Jr. (1989) Multiple hepatocyte-enriched nuclear factors function in the regulation of transthyretin and α_1-antitrypsin genes. *Mol. Cell. Biol.* **9**, 1415-1425.

Courey AJ, Holtzmann DA, Jackson SP, Tjian R. (1989) Synergistic activation by the glutamine-rich domains of the human transcription factor Sp1. *Cell* **59**, 927-836.

Creasey AA, Yamamoto R, Vitt CR. (1987) A high molecular weight component of the human TNF-R is associated with cytotoxicity. *Proc. Natl. Acad. Sci. USA* **84**, 3293-3297.

Cross SL, Halden NF, Lenardo MJ, Leonard WJ. (1989) Functionally distinct NFκB binding sites in the Ig *K* and IL2Rα chain genes. *Science* **244**, 466-469.

Cuturi MC, Murphy M, Costa-Giomi MP, Weinmann R, Perussia B, Trinchieri G. (1987) Independent regulation of TNF and lymphotoxin production by human peripheral blood lymphocytes *J. Exp. Med.* **165**, 1581-1594.

Czarniecki CW, Fennie CW, Powers DB, Estell DA. (1984) Synergistic antiviral and antiproliferative activities of *E. coli*-derived human α, ß and γ-interferons. *J. Virol.* **49**, 490-496.

Daniels DR, Deininger PL. (1985) Integration site preferences of the *Alu* family and similar repetitive DNA sequences. *Nucl. Acids Research* **13**, 8939-8954.

Danielsen M, Hinck L, Ringold GM. (1989) Two amino acids in the knuckle of the first zinc finger specify DNA response element activation by the glucocorticoid receptor. *Cell* **57**, 1131-1138.

Darlington GJ, Wilson DR, Lachman LB. (1986) Monocyte-conditioned medium, IL-1, and TNF stimulate the acute phase response in human hepatoma cells *in vitro*. *J. Cell. Biol.* **103**, 787-793.

Davis RL, Cheng P-F, Lassar AB, Weintraub H. (1990) The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. *Cell* **60**, 733-746.

Davis RL, Weintraub H, Lassar AB. (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**, 987-1000.

Debanne MT, Bell R, Dolovich J. (1976) Characteristics of the macrophage uptake of protease-α-macroglobulin complexes. *Biochem. Biophys. Acta* **428**, 466-475.

de Beer FC, Baltz ML, Munn EA, Feinstein A, Taylor J, Bruton C, Clamp JR, Pepys MB. (1982a) Isolation and characterization of CRP and SAP component in the rat. *Immunol.* **45**, 55-70.

de Beer FC, Hind CRK, Fox KM, Allan RM, Maseri A, Pepys MB. (1982b) Measurement of serum CRP concentration in myocardial ischaemia and infarction. *Br. Heart J.* **47**, 239-243.

De Li Shi, Savona C, Gagnon J, Cochet C, Chambaz EM, Feige J-J. (1990) TGFß stimulates the expression of α_2-macroglobulin by cultured bovine adrenocortical cells. *J. Biol. Chem.* **265**, 2881-2887.

Dente L, Ciliberto G, Cortese R. (1985) Structure of the human α_1-acid glycoprotein gene: sequence homology with other human acute phase genes. *Nucl. Acids Research* **13**, 3941-3952.

Dente L, Rither U, Tripodi M, Wagner EF, Cortese R. (1988) Expression of human alpha$_1$-AGP genes in cultured cells and in transgenic mice. *Genes Dev.* **2**, 259-266.

DePonti-Zilli L, Seiler-Tuyns A, Paterson BM. (1988) A 40bp sequence in the 3' end of the ß-actin gene regulates ß-actin mRNA transcription during myogenesis. *Proc. Natl. Acad. Sci. USA* **85**, 1389-1393.

Derynck R, Jarret JA, Chen EY, Eaton DH, Bell JR, Assoian RK, Roberts AB, Sporn MB, Goeddel DV. (1985) Human TGFß cDNA sequence and expression in normal amd transformed cells. *Nature* **316**, 701-705.

Descombes P, Chojkier M, Lichtsteiner S, Falvey E, Schibler U. (1990) LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. *Genes Dev.* **4**, 1541-1551.

Dhawan S, Fields RL, Robey, RA. (1990) A novel peptide from amyloid P component supports cell attachment. *Biochem. Biophys. Res. Comm.* **171**, 1284-1290.

Diamond MI, Miner JN, Yoshinaga SK, Yamamoto KR. (1990) Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. *Science* **249**, 1266-1272.

di Giovine FS, Duff GW. (1990) IL1: The first interleukin. *Immunol. Today* **11**, 13-20.

Dignam JD, Lebovitz RM, Roeder, R.G. (1983) Accurate transcription initiation by RNA polII in a soluble extract from isolated mammalian nuclei. *Nucl. Acids Research* **11**, 1475-1489.

Dinarello CA. (1984) Interleukin-1. *Rev. Infect. Dis.* **6**, 51-95.

Dinarello CA. (1989) Interleukin-1 and its biologically related cytokines. *Adv. Immunol.* **44**, 153-205.

Dinarello CA, Cannon JG, Wolff SM, Bernheim HA, Beutler B, Cerami A, Figari IS, Palladino MA Jr, O'Connor JV. (1986) TNF (cachectin) is an endogenous pyrogen and induces production of IL1. *J. Exp. Med.* **163**, 1433-1450.

Dinarello CA, Clark BD, Puren AJ, Savage N, Rosoff PM. (1989) The IL1-receptor. *Immunol. Today* **10**, 49-51.

Dinarello CA, Ikejima T, Warner SJC, Orencole SF, Lonnemann G, Cannon JG, Libby P. (1987) IL1 induces IL1: I. Induction of circulating IL1 in rabbits *in vivo* and in human mononuclear cells *in vitro*. *J. Immunol.* **139**, 1902-1910.

Dixit VM, Marks RM, Sarma V, Prochownik EV. (1989) The antimitogenic action of TNF is associated with increased AP-1/c-*jun* proto-oncogene transcription. *J. Biol. Chem.* **264**, 16905-16909.

Doolittle RF. (1980) Similar amino acid sequences: Chance or common ancestory? *Science* **214**, 149-159.

Dover G. (1989) Slips, strings and species. *Trends Genet.* **5**, 100-102.

Dower SK, Kronheim SR, March CJ, Conlon PJ, Hopp TP, Gillis S, Urdal DL. (1985) Detection and characterization of high-affinity plasma membrane receptors for human IL1. *J. Exp. Med.* **162**, 501-515.

Dower SK, Kronheim SR, Hopp TP, Cantrell M, Delley M, Gillis S, Henney CS, Urdal DL. (1986) The cell surface receptors for IL1α and IL1ß are identical. *Nature* **324**, 266-268.

Dowton SB, McGrew SD. (1990) Rat serum amyloid P component. *Biochem. J.* **270**, 553-556.

Dowton SB, Waggoner DJ. (1989) Armenian hamster female protein (serum amyloid P component) *J. Immunol.* **143**, 3776-3780.

Dowton SB, Woods DE, Mantzouranis EC, Colten HR. (1985) Syrian hamster female protein: analysis of female protein primary structure and gene expression. *Science* **228**, 1206-1208.

DuClos TW. (1989) C-reactive protein reacts with the U1 small nuclear ribonucleoprotein. *J. Immunol.* **143**, 2553-2559.

DuClos TW, Zlock LT, Rubin RL. (1988) Analysis of the binding of C-reactive protein to histones and chromatin. *J. Immunol.* **141**, 4266-4270.

Duh EJ, Maury WJ, Folks TM, Fauci AS, Rabson AB. (1989) TNFα activates HIV type I through induction of nuclear factor binding to the NFκB sites in the long terminal repeat. *Proc. Natl. Acad. Sci. USA* **86**, 5974-5978.

Dwulet FE, Wallace DK, Benson MD. (1988) Amino acid structures of multiple forms of amyloid-related serum protein SAA from a single individual. *Biochemistry* **27**, 1677-1682.

Dyck RF, Lockwood CM, Kershaw M, McHugh N, Duance VC, Baltz ML, Pepys MB. (1980) Amyloid P component is a constituent of normal human glomerula basement membrane. *J. Exp. Med.* **152**, 1162-1174.

Edbrooke MR, Burt DW, Cheshire JK, Woo P. (1989) Identification of *cis*-acting sequences responsible for phorbol ester induction of human serum amyloid A gene expression via a nuclear factor κB-like transcription factor. *Mol. Cell. Biol.* **9**, 1908-1916.

Edbrooke MR, Foldi J, Li F, Cheshire JK, Faulkes D, Woo P. (1991) Constitutive and NFκB-like proteins in the regulation of the SAA gene by IL1. *Cytokine* In press.

Edbrooke MR, Woo P. (1989) Regulation of human SAA gene expression by cytokines. *In* "Acute phase proteins and the acute phase response" (Pepys MB, ed.) Springer-Verlag, London, pp. 21-27.

Eickbush TH, Burke WD. (1986) The silkmoth late chorion locus II. Gradients of gene conversion in two paired multigene families. *J. Mol. Biol.* **190**, 357-366.

Eisenberg SP, Evans RJ, Arend WP, Verderber E, Brewer MT, Hannum CH, Thompson RC. (1990) Primary structure and functional expression from cDNA of a human IL-1 receptor antagonist. *Nature* 343, 341-346.

Elias JA, Lentz V. (1990) IL1 and TNF synergistically stimulate fibroblast IL6 production and stabilize IL6 mRNA. *J. Immunol.* **145**, 161-166.

Elias JA, Trinchieri G, Beck JM, Simon PL, Sehgal PB, May LT, Kern JA. (1989a) A synergistic interaction of IL1 and IL6 mediates the thymocyte stimulating activity produced by rIL1-stimulated fibroblasts. *J. Immunol.* **142**, 509-514.

Elias JA, Reynolds MM, Kotloff KM, Kern JA. (1989b) Fibroblast IL1ß: synergistic stimulation by recombinant IL1 and TNF and posttranscriptional regulation. *Proc. Natl. Acad. Sci. USA* **86**, 6171-6175.

Emilie D, Crevon MC, Auffredou MT, Galanaud P. (1988) Glucocorticoid-dependent synergy between IL1 and IL6 for human B lymphocyte differentiation. *Eur. J. Immunol.* **18**, 2043-2047.

Engelmann H, Holtmann H, Brakebusch C, Avni YS, Sarov I, Nophar Y, Hadas E, Leitner O, Wallach D. (1990) Antibodies to a soluble form of TNF-R have TNF-like activity. *J. Biol. Chem.* **265**, 14497-14504.

Engler R. (1982) Recent findings on the biological role of haptoglobin in rats. *In* "Marker proteins in inflammation" (R.C. Allen *et al*, eds.) Walter de Gruyter, Berlin. pp. 209-213.

Ericsson LH, Eriksen N, Walsh KA, Benditt EP. (1987) Primary structure of duck amyloid protein A. *FEBS Lett.* **218**, 11-16.

Esnard F, Gauthier F. (1983) Rat α_1CPI. An acute phase protein identical with alpha$_1$ acute phase globulin. *J. Biol. Chem.* **258**, 12443-12447.

Esparza I, Mannel D, Ruppel A, Falk W, Krammer PH. (1987) IFNγ and lymphotoxin or TNF act synergistically to induce macrophage killing of tumour cells and Schistosomula of *Schistosoma mansoni*. *J. Exp. Med.* **166**, 589-594.

Espel E, Fromental C, Reichenbach P, Nabholz M. (1990) Activity and IL1 responsiveness of SV40 enhancer motifs in a rodent immature T cell line. *EMBO J.* **9**, 929-937.

Evans E, Courtois GM, Kilian PL, Fuller GM, Crabtree GR. (1987) Induction of fibrinogen and a subset of acute phase response genes involves a novel monokine which is mimicked by phorbol esters. *J. Biol. Chem.* **262**, 10850-10854.

Falvey E, Schibler U. (1991) How are the regulators regulated? *FASEB J.* **5**, 309-314.

Fan C-M, Maniatis T (1990) A DNA binding protein containing two widely separated zinc finger motifs that recognize the same sequence. *Genes Dev.* **4**, 29-42.

Feinberg AP, Vogelstein B. (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6-13.

Felsenstein J. (1990) PHYLIP Manual Version 3.3. University Herbarium, University of California, Berkeley, California.

Fenton MJ, Clark BD, Collins KL, Webb AC, Rich A, Auron PE. (1987) Transcriptional regulation of the human prointerleukin 1ß gene. *J. Immunol.* **138**, 3972-3979.

Fey GH, Fuller GM. (1987) Regulation of acute phase gene expression by inflammatory mediators. *Mol. Biol. Med.* **4**, 323-338.

Fey GH, Gauldie J. (1990) The acute phase response of the liver in inflammation. *Prog. Liver. Dis.* **9**, 89-116.

Fink GR. (1986) Translational control of transcription in eukaryotes. *Cell* **45**, 155-156.

Finnegan DJ. (1989) Eukaryotic transposable elements and genome evolution. *Trends Genet.* **5**, 103-107.

Fitch DHA, Mainone C, C, Goodman M, Slightom JL. (1990) Molecular history of gene conversions in the primate fetal γ-globin genes. *J. Biol. Chem.* **265**, 781-793.

Flanagan PM, Kelleher III RJ, Sayre MH, Tschochner H, Kornberg RD. (1991) A mediator required for activation of RNA polymerase II transcription *in vitro*. *Nature* **350**, 436-438.

Fowlkes DM, Mullis NT, Comeau CM, Crabtree GR. (1984) Potential basis for regulation of the coordinately expressed fibrinogen genes: homology in the 5' flanking regions. *Proc. Natl. Acad. Sci. USA* **81**, 2313-2316.

Frain M, Swart G, Monaci P, Nicosia A, Stampfil S, Frank R, Cortese R. (1989) The liver-specific transcription factor LF-B1 contains a highly diverged homeobox DNA binding domain. *Cell* **59**, 145-157.

Frampton J, Walker M, Plumb M, Harrison PR. (1990) Synergy between the NF-E1 erythroid-specific transcription factor and the CACCC factor in the erythroid specific promoter of the human porphobilinogen deaminase gene. *Mol. Cell. Biol.* **10**, 3838-3842.

Frankel AD, Kim PS. (1991) Modular structure of transcription factors: implications for gene regulation. *Cell* **65**, 717-719.

Freimuth WW, Depper JM, Nabel GJ. (1989) Regulation of the IL2Rα gene: interaction of a κB binding protein with cell specific transcription factors. *J. Immunol.* **143**, 3064-3068.

Friedman MJ. (1983) Control of malaria virulence by α_1AGP (orosomucoid), an acute phase (inflammatory) reactant. *Proc. Natl. Acad. Sci. USA* **80**, 5421-5424.

Fuller GM, Otto JM, Woloski BM, McGray CT, Adams MA. (1985) The effects of hepatocyte stimulating factor on fibrinogen biosynthesis in hepatocyte monolayers. *J. Cell. Biol.* **101**, 1481-1486.

Fung W-P, Schreiber G. (1987) Structure and expression of the genes for major acute phase α_1-protein (thiostatin) and kininogen in the rat. *J. Biol. Chem.* **262**, 9298-9308.

Furutani Y, Notake M, Fuki T, Ohue M, Nomura H, Yamada M, Nakamura S. (1986) Complete nucleotide sequence of the gene for human IL1α. *Nucl. Acids Research* **14**, 3167-3179.

Gallis B, Prickett KS, Jackson J, Slack J, Schooley K, Sims JE, Dower SK. (1989) IL1 induces rapid phosphorylation of the IL1 receptor. *J. Immunol.* **143**, 3235-3240.

Ganapathi MK, May LT, Schultz D, Brabenec A, Weinstein J, Sehgal PB, Kushner I. (1988a) Role of interleukin-6 in regulating synthesis of C-reactive protein and serum amyloid A in human hepatoma cell lines. *Biochem. Biophys. Res. Comm.* **157**, 271-277.

Ganapathi MK, Schultz D, Mackiewicz A, Samols D, Hu S-I, Brabenec A, Macintyre SS, Kushner I. (1988b) Heterogeneous nature of the acute phase response. *J. Immunol.* **141**, 564-569.

Ganter U, Arcone R, Toniatti C, Morrone G, Ciliberto G. (1989) Dual control of C-reactive protein gene expression by interleukin-1 and interleukin-6. *EMBO J.* **8**, 3773-3779.

Garcia-Welsh A, Schneiderman JS, Baly DL. (1990) IL-1 stimulates glucose transport in rat adipose cells. Evidence for receptor discrimination between IL1ß and IL1α. *FEBS Lett.* **269**, 421-424.

Gauldie J. (1989) Interleukin 1 in the acute phase response. *In* "Acute phase proteins in the acute phase response" (Pepys MB, ed.). Springer-Verlag, London, pp. 1-20.

Gauldie J, Northemann W, Fey GH. (1990) IL-6 functions as an exocrine hormone in inflammation. *J. Immunol.* **144**, 3804-3808.

Gauldie J, Richards C, Harnish D, Lansdorp P, Baumann H. (1987) Interferon $ß_2$/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. *Proc. Natl. Acad. Sci. USA* **84**, 7251-7255.

Gearing DP, Gough NM, King JA, Hilton DJ, Nicola NA, Simpson RJ, Nice EC, Kelso A, Metcalf D. (1987) Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF) *EMBO J.* **6**, 3995-4002.

Gehring MR, Shiels BR, Northemann W, de Bruijn MHL, Kan C-C, Chain AC, Noonan DJ, Fey GH. (1987) Sequence of rat liver α_2-macroglobulin and acute phase control of its messenger RNA. *J. Biol. Chem.* **262**, 446-454.

Gehring WJ. (1987) Homeoboxes in the study of development. *Science* **236**, 1245-1252.

Geiger T, Andus T, Klapproth J, Northoff H, Heinrich PC. (1988) Induction of α_1-acid glycoprotein by recombinant human interleukin-1 in rat hepatoma cells. *J. Biol. Chem.* **263**, 7141-7146.

Gentz R, Rauscher III FJ, Abate C, Curran T. (1989) Parallel association of Fos and Jun leucine zippers juxtaposes DNA binding domains. *Science* **243**, 1695-1699.

Gervais C, Suh M. (1990) Serum amyloid A protein-related mRNA expression in herpes simplex virus type 2-transformed hamster cells. *Mol. Cell. Biol.* **10**, 4412-4414.

Ghosh S, Baltimore D. (1990) Activation *in vitro* of NFκB by phosphorylation of its inhibitor IκB. *Nature* **344**, 678-682.

Ghosh S, Gifford AM, Riviere LR, Tempst P, Nolan GP, Baltimore D. (1990) Cloning of the p50 DNA binding subunit of NFκB: homolgy to *rel* and *dorsal*. *Cell* **62**, 1019-1029.

Gilbert W. (1978) Why genes in pieces? *Nature* **271**, 501.

Gilbert W. (1985) Genes-in-pieces-revisited. *Science* **228**, 823-824.

Gill G, Ptashne M. (1988) Negative effect of the transcriptional activator GAL4. *Nature* **334**, 721-724.

Gilmore TD. (1990) NFκB, KBF1, *dorsal*, and *rel*ated matters. *Cell* **62**, 841-843.

Giniger E, Ptashne M. (1987) Transcription in yeast activated by a putative amphipathic α helix linked to a DNA binding unit. *Nature* **330**, 670-672.

Gitlin JD. (1988) Transcriptional regulation of ceruloplasmin gene expression during inflammation. *J. Biol. Chem.* **263**, 6281-6287.

Gius D, Cao X, Rauscher III FJ, Cohen DR, Curran T, Sukhatme VP. (1990) Transcriptional activation and repression by Fos are independent functions. *Mol. Cell. Biol.* **10**, 4243-4255.

Glenner GG, Terry W, Harada M, Isersky CD, Page D. (1971) Amyloid fibril proteins: proof of homology with immunoglobulin light chains by sequence analyses. *Science* **172**, 1150-1151.

Glibetic MD, Baumann H. (1986) Influence of chronic inflammation on the level of mRNA for acute-phase reactants in the mouse liver. *J. Immunol.*, **137**, 1616-1622.

Glover DM. (1985) DNA cloning, a practical approach. Vol V, p121.

Goldberger G, Bing GH, Sipe JD, Rits M, Colten HR. (1987) Transcriptional regulation of genes encoding the acute-phase proteins CRP, SAA, and C3. *J. Immunol.* **138**, 3967-3971.

Goldfarb D, Nathan I, Dvilansky A, Khoda G, Shainkin-Kestenbaum R. (1990) Modulation of transformed cells by SAA. *In* VIth International Symposium on Amyloidosis: Programme and Abstracts, p.31.

Goldman ND, Liu TY. (1987) Biosynthesis of human CRP in cultured hepatoma cells is induced by a monocyte factor(s) other than IL1. *J. Biol. Chem.* **262**, 2363-2368.

Goldstein IM, Kaplan HB, Edelson HS, Weissmann G. (1979) Ceruloplasmin. A scavenger of superoxide anion radicals. *J. Biol. Chem.* **254**, 4040-4045.

Gollaher CJ, Bausserman LL. (1989) Hepatic catabolism of SAA during an acute phase response and chronic inflammation. *Proc. Soc. Exp. Biol. Med.* **194**, 245-250.

Gorevic PD, Rosenthal CJ, Franklin EC. (1976) Amyloid-related serum component (SAA): studies in acute infections, medullary thyroid carcinoma and post-surgery. *Clin. Immunol. Immunopathol.* **6**, 83-93.

Gorman CM, Moffat LF, Howard BH. (1982) Recombinant genomes which express CAT in mammalian cells. *Mol. Cell. Biol.* **2**, 1044-1051.

Gotschlich E, Stetson CA Jr. (1960) Immunologic cross-reaction among mammalian acute phase proteins. *J. Exp. Med.* **111**, 441-451.

Graham FL, van der Eb AJ. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**, 456-467.

Graves BJ, Johnson PF, McKnight SL. (1987) Homologous recognition of a promoter domain common to the MSV LTR and HSV *tk* gene. *Cell* **44**, 565-576.

Gray PW, Barrett K, Chantry D, Turner M, Feldmann M. (1990) Cloning of human TNF-receptor cDNA and expression of recombinant soluble TNF-binding protein. *Proc. Natl. Acad. Sci. USA* **87**, 7380-7384.

Greeley NH, Murali MR, Martin ME, Josephson AS, Rosenthal MD. (1987) SAA protein inhibits the inflammatory response of activated phagocytes. *J. Allerg. Clin.* **79**, 158. (Abstract).

Grenfell S, Smithers N, Miller K, Solari R. (1989) Receptor-mediated endocytosis and nuclear transport of human IL1α. *Biochem. J.* **264**, 813-822.

Grey I, Gershon RK, Waksman BH. (1972) Potentiation of the T lymphocyte response to mitogens. I. The responding cell. *J. Exp. Med.* **136**, 128-138.

Groot PC, Bleeker MJ, Pronk JC, Arwert F, Mager WH, Planta RJ, Eriksson, AW, Frants RR. (1989) The human α-amylase multigene family consists of haplotypes with variable numbers of genes. *Genomics* **5**, 29-42.

Guillouzo A, Delers F, Clement B, Bernard M, Engler R. (1984) Long term production of acute phase proteins by adult rat hepatocytes co-cultured with another liver cell-type. *Biochem. Biophys. Res. Comm.* **120**, 311-317.

Gutman A, Wasylyk B. (1990) The collagenase gene promoter contains a TPA and oncogene-resposive unit encompassing the PEA3 and AP1 sites. *EMBO J.* **9**, 2241-2246.

Haegeman G, Content J, Volckaert G, Derynck R, Tavernier J, Fiers W. (1986) Structural analysis of the sequence coding for an inducible 26-kDa protein in human fibroblasts. *Eur. J. Biochem.* **159**, 625-632.

Han H-M, Kolhatkar AA, Marino MW, Manchester KM, Donner DB. (1990) Identification, characterization, and homologous up-regulation of latent (cryptic) receptors for TNFα in rat liver plasma membranes. *J. Biol. Chem.* **265**, 18590-18594.

Hanahan D, Meselson M. (1983) Plasmid screening at high colony density. *Gene* **10**, 63-67.

Hannum CH, Wilcox CJ, Arend WP, Joslin FG, Dripps DJ, Heimdal PL, Armes LG, Sommer A, Eisenberg SP, Thompson RC. (1990) IL-1 receptor antagonist activity of a human IL-1 inhibitor. *Nature* **343**, 336-340.

Harats N, DiBartola SP, Benson MD, Kluve-Beckerman B. (1991) Abyssinian cat model for AA amyloidosis: SAA gene analysis. *In* "Amyloid and Amyloidosis 1990" (Natvig JB, *et al*, eds.) Kluwer Academic Publishers, Dordrecht. pp. 32-35.

Hardon EM, Frain M, Paonessa G, Cortese R. (1988) Two distinct factors interact with the promoter regions of several liver-specific genes. *EMBO J.* **7**, 1711-1719.

Hastie ND. (1989) Highly repeated DNA families in the genome of *Mus musculus*. *In* "Genetic variants and strains of the laboratory mouse", (Lyon MF, Searle AG, eds.) Oxford University Press, pp. 559-573.

Hattori M, Abraham LJ, Fey GH. (1989a) Identification of an interleukin 6 response element in the rat α_2-macroglobulin gene. *Ann. N. Y. Acad. Sci.* **537**,

Hattori M, Kusakabe S-i, Ohgusu H, Tsuchiya Y, Ito T, Sakaki Y. (1989b) Structure of the rat α_2-macroglobulin-coding gene. *Gene* **77**, 333-340.

Hattori M, Abraham LJ, Northemann W, Fey, GH. (1990) Acute phase response induces a specific complex between hepatic nuclear proteins and the IL6RE of the rat α_2M gene. *Proc. Natl. Acad. Sci. USA* **87**, 2364-2368.

Hattori M, Sakaki Y. (1986) Dideoxy sequencing method using denatured plasmid templates. *Anal. Biochem.* **152**, 232-238.

Hayashi S, Scott MP. (1990) What determines the specificity of action of *Drosophila* homeodomain proteins? *Cell* **63**, 883-894.

Hazuda DJ, Strickler J, Kueppers F, Simon PL, Young PR. (1990) Processing of precursor IL1ß and inflammatory disease. *J. Biol. Chem.* **265**, 6318-6322.

Hebert L, Gervais F. (1990) Apo-SAA1/apo-SAA2 isotype ratios during casein- and amyloid-enhancing-factor-induced secondary amyloidosis in A/J and C57BL/6J mice. *Scand. J. Immunol.* **31**, 167-173.

Heinrich PC, Castell JV, Andus T. (1990) Interleukin-6 and the acute phase response. *Biochem. J.* **265**, 621-636.

Helle M, Brakenhof JPJ, De Groot ER, Aarden LA. (1988) IL6 is involved in IL1-induced activities. *Eur. J. Immunol.* **18**, 957-959.

Hellu RA, Song K, Onasch MA, Fisher WH, Chang D, Ringold GM. (1990) cDNA cloning of a receptor for TNF and demonstration of a shed form of the receptor. *Proc. Natl. Acad. Sci. USA* **87**, 6151-6155.

Hensel G, Meichle A, Hizenmaier K, Kronke M. (1989) PMA-responsive 5' flanking sequences of the human TNF gene. *Lymphokine Res.* **8**, 347-351.

Hibi M, Murakami M, Saito M, Hirano T, Taga T, Kishimoto T. (1990) Molecular cloning and expression of an IL-6 signal transducer, gp130. *Cell* **63**, 1149-1157.

Higgins DG, Sharp PM. (1988) Clustal: a package for performing multiple sequence alignment on a microcomputer. *Gene* **73**, 237-244.

Hill MR, Stith RD, McCallum RE. (1986) IL1: a regulatory role in glucocorticoid-regulated hepatic metabolism. *J. Immunol.* **137**, 858-862.

Hill MR, Stith RD, McCallum RE. (1988) Human recombinant IL1 alters glucocorticoid-receptor function in Reuber hepatoma cells. *J. Immunol.* **141**, 1522-1528.

Hirano T, Teranishi T, Lin B, Onone K. (1984) Human helper T cell factor(s). IV. Demonstration of a human late-acting B cell differentiation factor acting on *Staphylococcus aureus* Cowan I-stimulated B cells. *J. Immunol.* **133**, 798-802.

Hoeffler JP, Meyer TE, Yun Y, Jameson JL, Habener JF. (1988) CRE DNA binding protein: structure based on a cloned placental DNA. *Science* **242**, 1430-1433.

Hoffman JS, Ericsson LH, Eriksen N, Walsh KA, Benditt EP. (1984) Murine tissue amyloid protein AA. NH_2-terminal sequence identity with only one of two serum amyloid protein (apoSAA) gene products. *J. Exp. Med.* **159**, 641-646.

Hofmann JF-X, Laroche T, Brand AH, Gasser SM. (1989) RAP-1 factor is necessary for DNA loop formation *in vitro* at the silent mating type locus *HML*. *Cell* **57**, 725-737.

Hohmann H-P, Remy R, Poschl B, van Loon APGM. (1990) TNFs-α and ß bind to the same two types of TNF-Rs and maximally activate the transcription factor NFκB at low receptor occupancy. *J. Biol. Chem.* **265**, 15183-15188.

Hollenberg SM, Evans RM. (1988) Multiple and cooperative *trans*-activation domains of the human glucocorticoid receptor. *Cell* **55**, 899-906.

Holsti MA, Raulet DH. (1989) IL6 and IL1 synergize to stimulate IL2 production and proliferation of peripheral T cells. *J. Immunol.* **143**, 3514-2519.

Hong GF. (1987) The use of DNase1, buffer gradient gel and ^{35}S label for DNA sequencing. *Methods Enzymol.* **155**, 93-110.

Hope IA, Mahadevan S, Struhl K (1988) Structural and functional characterization of the short acidic transcriptional activation region of yeast GCN4 protein. *Nature* **333**, 635-640.

Horuk R, Huang JJ, Covington M, Newton RC. (1987) A biochemical and kinetic analysis of the IL1-R. Evidence for differences in molecular properties of IL1-Rs. *J. Biol. Chem.* **262**, 16275-16278.

Houssiau FA, Coulie PG, Olive D, Van Snick J. (1988) Synergistic activation of human T cells by IL1 and IL6. *Eur. J. Immunol.* **18**, 653-656.

Hoyos B, Ballard DW, Bohnlein E, Siekevitz M, Greene WC. (1989) Kappa-B specific DNA binding proteins: role in the regulation of human IL2 gene expression. *Science* **244**, 457-460.

Hu S-I, Macintyre SM, Schultz D, Kushner I, Samols D. (1988) Secretion of rabbit CRP by transfected human cell lines is more rapid than by cultured rabbit hepatocytes. *J. Biol. Chem.* **263**, 1500-1504.

Huang JH, Rienhoff HY Jr, Liao WSL. (1990) Regulation of mouse serum amyloid A gene expression in transfected hepatoma cells. *Mol. Cell. Biol.* **10**, 3619-3625.

Huber P, Laurent M, Dalmon J. (1990) Human ß-fibrinogen gene expression. *J. Biol. Chem.* **265**, 5695-5701.

Hulkower KI, Georgescu HI, Evans CH. (1989) Altered patterns of protein phosphorylation in articular chondrocytes treated with interleukin-1 or synovial cytokines. *FEBS Lett.* **257**, 228-232.

Hurliman J, Thorbecke GJ, Hochwald GM. (1966) The liver as the site of CRP formation. *J. Exp. Med.* **123**, 365-378.

Husby G, Natvig JB. (1974) A serum component related to nonimmunoglobulin amyloid protein A, a possible precursos of the fibrils. *J. Clin. Invest.* **53**, 1054-1061.

Husby G, Sletten K. (1986) Chemical and clinical classification of amyloidosis 1985. *Scand. J. Immunol.* **23**, 253-265.

Husby G, Sletten K, Michaelsen TE, Natvig JB. (1972) Alternative, non-immunoglobulin origin of amyloid fibrils. *Nature New Biol.* **238**, 187.

Husebekk A, Skogen B, Husby G, Marhaug G. (1985) Transformation of amyloid precursor SAA to protein AA and incorporation in amyloid fibrils *in vivo*. *Scand. J. Immunol.* **21**, 283-287.

Hwu HR, Roberts JW, Davidson EH, Britten RJ. (1986) Insertion and/or deletion of many repeated DNA sequences in human and higher ape evolution. *Proc. Natl. Acad. Sci. USA* **83**, 3875-3879.

Ikebuchi K, Wong GG, Clark SC, Ihle JN, Hirai Y, Ogawa M. (1987) IL6 enhancement of IL3-dependent proliferation of multipotential hemopoietic progenitors. *Proc. Natl. Acad. Sci. USA* **84**, 9035-9039.

Imamura K, Shremann ML, Spriggs D, Kufe D. (1987) Expression of TNF-Rs on human monocytes and internalization of receptor bound ligand. *J. Immunol.* **139**, 2989-2992.

Irvine RF. (1982) How is the level of free arachidonic acid controlled in mammalian cells. *Biochem. J.* **204**, 3-16.

Ish-Horowicz D; Burke JF. (1981) Rapid and efficient cosmid cloning. *Nucl. Acids Research* **9**, 2989-2998.

Israel A, LeBail O, Hatat D, Piette J, Kieran M, Logeat F, Wallach D, Fellous M, Kourilsky P. (1989) TNF stimulates expression of mouse MHC class I genes by inducing an NFκB-like enhancer binding activity which displaces constitutive factors. *EMBO J.* **8**, 3793-3800.

Isshiki H, Akira S, Tanabe O, Nakajima T, Shimamoto T, Hirano T, Kishimoto T. (1990) Constitutive and IL-1-inducible factors interact with the IL-1-resonsive element in the IL-6 gene. *Mol. ell. Biol.* **10**, 2757-2764.

James K. (1990) Interactions between cytokines and α_2-macroglobulin. *Immunol. Today*, **11**, 163-166.

Janson L, Pettersson U. (1990) Cooperative interactions between transcription factors Sp1 and OTF-1. *Proc. Natl. Acad. Sci. USA* **87**, 4732-4736.

Jarman AP, Wells RA (1989) Hypervariable minisatellites: recombinators or innocent bystanders? *Trends Genet.* **5**, 367-371.

Jeffreys AJ, Wilson V, Thein SL. (1985) Hypervariable "minisatellite" regions in human DNA. *Nature* **314**, 67-73.

Johnson PF, Landschulz WH, Graves BJ, McKnight SL. (1987) Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. *Genes Dev.* **1**, 133-146.

Jonat C, Rahmsdorf HJ, Park K-K, Cato ACB, Gebel S, Ponta H, Herrlich P. (1990) Antitumour promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell* **62**, 1189-1204.

Jones N. (1990) Transcriptional regulation by dimerization: two sides to an incestuous relationship. *Cell* **61**, 9-11.

Jurka J, Smith T. (1988) A fundamental division in the *Alu* family of repeated sequences. *Proc. Natl. Acad. Sci. USA* **85**, 4775-4778.

Kadonaga JT, Carner KR, Masiarz FR, Tjian R. (1987) Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell* **51**, 1079-1090.

Kageyama R, Kitamura N, Ohkubo H, Nakanishi S. (1987) Differing utilization of homologous transcription initiation sites of rat K and T kininogen genes under inflammation condition. *J. Biol. Chem.* **262**, 2345-2351.

Kakidani H, Ptashne M. (1988) GAL4 activates gene expression in mammalian cells. *Cell* **52**, 161-167.

Kaplan AP, Silverberg M. (1988) Mediators of inflammation: an overveiw. *Meth. Enzymol.* **163**, 3-23.

Karim FD, Urness LD, Thummel CS, Klemsz MJ, McKercher SR, Celada A, Van Beveren C, Maki RA, Gunther CV, Nye JA, Graves BJ. (1990) The ETS-domain: a new DNA-binding motif that recognizes a purine-rich core DNA sequence. *Genes Dev.* **4**, 1451-1453.

Katsura PE, Gottschall PE, Akira A. (1988) Identification of a high-affinity receptor for IL-1ß in rat brain. *Biochem. Biophys. Res. Comm.* **156**, 61-67.

Kaur P, Saklatvala J. (1988) IL1 and TNF increase phosphorylation of fibroblast proteins. *FEBS Lett.* **241**, 6-10.

Kaur P, Welch WJ, Saklatvala J. (1989) IL1 and TNF increase phosphorylation of the small heat shock protein. Effects in fibroblasts, HepG2 and U937 cells. *FEBS Lett.* **258**, 269-273.

Keegan L, Gill G, Ptashne M. (1986) Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. *Science* **231**, 699-704.

Kelleher III RJ, Flanagan PM, Kormberg RD. (1990) A novel mediator between activator proteins and the RNA polII transcription apparatus. *Cell* **61**, 1209-1215.

Kerr LD, Miller DB, Matrisian LM. (1990) TGFß1 inhibition of transin/stromelysin gene expression is mediated through a Fos binding sequence. *Cell* **61**, 267-268.

Kester M, Simonson MS, Mene P, Sedor JR. (1989) IL1 generates transmembrane signals from phospholipoproteins through novel pathways in cultured rat mesangial cells. *J. Clin. Invest.* **83**, 718-723.

Keyeux G, Lefranc G, Lefranc M-P. (1989) A multigene deletion in the human IGH constant region locus involves highly homologous hotspots of recombination. *Genomics* **5**, 431-441.

Kieran M, Blank V, Logeat F, Vanderckhove J, Lottspeich F, LeBail O, Urban MB, Kourilsky P, Baeuerle PA, Israel A. (1990) The DNA binding subunit of NFκB is identical to KBF1 and homologous to the *rel* oncogene product. *Cell* **62**, 1007-1018.

Kioussis D, Wilson F, Daniels C, Leveton C, Taverne J, Playfair JHL. (1987) Expression and rescuing of a cloned human tumour necrosis factor gene using an EBV-based shuttle cosmid vector. *EMBO J.* **6**, 355-361.

Kishimoto T. (1989) The biology of IL-6. *Blood* **74**, 1-10.

Kishimoto T, Hirano T. (1988) Molecular regulation of the B lymphocyte response. *Annu. Rev. Immunol.* **6**, 485-512.

Klug A, Rhodes D. (1987) "Zinc fingers": a novel protein motif for nucleic acid recognition. *Trends Biochem. Sci.* **12**, 464-469.

Kluve-Beckerman B, Brinckerhoff C, Benson MD. (1991a) Sequence analysis of a third human SAA gene. *In* "Amyloid and Amyloidosis 1990", (Natvig JB, *et al*, eds.) Kluwer Academic Publishers, Dordrecht. pp. 24-27.

Kluve-Beckerman B, Liepnieks J, Benson MD. (1991b) Differential regulation of human SAA isoforms. *In*"Amyloid and Amyliodosis 1990", (Natvig JB, *et al*, eds.) Kluwer Academic Publishers, Dordrecht. pp. 125-128.

Kluve-Beckerman B, Long GL, Benson MD. (1986a) DNA sequence evidence for polymorphic forms of human serum amyloid A (SAA). *Biochem. Genet.* **24**, 795-803.

Kluve-Beckerman B, Naylor SL, Marshall A, Gardner JC, Shows TB, Benson MD. (1986b) Localization of human SAA gene(s) to chromosome 11 and detection of DNA polymorphisms. *Biochem. Biophys. Res. Comm.* **137**, 1196-1204.

Kluve-Beckerman B, Dwulet FE, Benson MD. (1988) Human serum amyloid A: three hepatic mRNAs and the corresponding proteins in one person. *J. Clin. Invest.* **82**, 1670-1675.

Kluve-Beckerman B, Dwulet FE, DiBartola SP, Benson MD. (1989) Primary structures of dog and cat amyloid A proteins: comparison to human AA. *Comp. Biochem. Physiol.* **94B**, 175-183.

Knudsen PJ, Dinarello CA, Strom TB. (1986) Prostaglandins posttranscriptionally inhibit monocyte expression of IL1 activity by increasing intracellular cAMP. *J. Immunol.* **137**, 3189-3194.

Kohase M, May LT, Tamm I, Vilcek J, Sehgal PB. (1987) A cytokine network in human diploid fibroblasts: interactions of ßIFNs, TNF, PDGF, and IL1. *Mol. Cell. Biol.* **7**, 273-280.

Koj A. (1974) Acute phase reactants. Their synthesis, turnover and biological significance. *In* "Structure and function of plasma proteins" (Allison AC, ed.) Plenum Press, London. Vol. I, pp. 73-125.

Koj A, Gauldie J, Regoeczi E, Sauder DN, Sweeney GD. (1984) The acute phase response of cultured rat hepatocytes. System characterization and the effect of human cytokines. *Biochem. J.* **224**, 505-514.

Koj A, Kurdowska A, Magielska-Zero D, Rokita H, Sipe JD, Dayer JM, Demczuk S, Gauldie J. (1987) Limited effects of recombinant human and murine IL1 and TNF on production of APPs by cultured rat hepatocytes. *Biochem. Int.* **14**, 553-560.

Kostura MJ, Tocci MJ, Limjuco G, Chin J, Cameron P, Hillman AG, Chartrain NA, Schmidt JA. (1989) Identification of a monocyte specific pre-IL1ß convertase activity. *Proc. Natl. Acad. Sci. USA* **86**, 5227-5231.

Kourilsky P. (1986) Molecular mechanisms for gene conversion in higher cells. *Trends Genet.* **2**, 60-63.

Kovacs EJ, Oppenheim JJ, Young HA. (1986) Induction of c-*fos* and c-*myc* expression in T lymphocytes after treatment with recombinant IL1α. *J. Immunol.* **137**, 3649-3651.

Kozak M. (1989) The scanning model for translation. An update. *J. Cell. Biol.* **108**, 229-241.

Kriegler M, Perez C, DeFay K, Albert I, Lu SD. (1988) A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. *Cell* **53**, 45-53.

Kroggel R, Martin M, Pingoud V, Dayer J-M, Resch K. (1988) Two-chain structure of the human IL1-R. *FEBS Lett.* **229**, 59-62.

Kudo S, Fukuda M. (1989) Structural organization of glycophorin A and B genes: glycophorin B gene evolved by homologous recombination at *Alu* repeat sequences. *Proc. Natl. Acad. Sci. USA* **86**, 4619-4623.

Kunz D, Zimmerman R, Heisig M, Heinrich PC. (1989) Identification of the promoter sequences involved in the IL6 dependent expression of the rat α_2M gene. *Nucl. Acids Research* **17**, 1121-1138.

Kushner I. (1982) The phenomenon of the acute phase response. *Ann. N. Y. Acad. Sci.* **389**, 39-48.

Kushner I. (1988) The acute phase response: an overview. *Meth. Enzymol.* **163**, 373-383.

Kushner I, Kaplan MH. (1961) Studies of acute phase proteins I. An immunohistochemical method for the localization of CxRP in rabbits. Association with necrosis in local inflammatory lesions. *J. Exp. Med.* **114**, 961-973.

Labuda D, Striker T. (1988) Sequence conservation in *Alu* evolution. *Nucl. Acids Research* **17**, 2477-2491.

Lai E, Prezioso VR, Smith E, Litvin O, Costa RH, Darnell JE Jr. (1990) HNF-3A, a hepatocyte enriched transcription factor of novel structure is regulated transcriptionally. *Genes Dev.* **4**, 1427-1436.

Landschulz WH, Johnson PF, McKnight SL. (1988) The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* **240**, 1759-1764.

Landschulz WH, Johnson PF, McKnight SL. (1989) The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. *Science*, **243**, 1681-1688.

Larrick JW. (1989) Native IL-1 inhibitors. *Immunol. Today* **10**, 61-66.

Latchman DS. (1990) Eukaryotic transcription factors. *Biochem. J.* **270**, 281-289.

Latimer JL, Berger FG, Baumann H. (1990) Highly conserved upstream regions of the α_1AT gene in two mouse species govern liver-specific expression by different mechanisms. *Mol. Cell. Biol.* **10**, 760-769.

Lavie G, Zucker-Franklin D, Franklin EC. (1980) Elastase-type proteases on the surface of human blood monocytes: possible role in amyloid formation. *J. Immunol.* **125**, 175-180.

Le J, Vilcek J. (1987) Biology of disease: TNF and IL1: cytokines with multiple overlapping biological activities. *Lab. Invest.* **56**, 234-248.

Le K-J, Liu T, Zon G, Soravia E, Liu T-Y, Goldman ND. (1985) Genomic DNA sequence for human CRP. *J. Biol. Chem.* **260**, 13377-13383.

Le PT, Mortensen RF. (1986) Induction and regulation by monokines of hepatic synthesis of the mouse SAP component. *J. Immunol.* **136**, 2526-2533.

Leblanc J-F, Cohen L, Rodrigues M, Hiscott J. (1990) Synergism between distinct enhanson domains in viral induction of the human beta interferon gene. *Mol. Cell. Biol.* **10**, 3987-3993.

LeBowitz JH, Clerc RG, Brenowitz M, Sharp PA. (1989) The Oct-2 protein binds cooperatively to adjacent octamer sites. *Genes Dev.* **3**, 1625-1638.

Lehrmann MA, Schneider WJ, SUdhof TC, Brown MS, Goldstein JL, Russell DW. (1985) Mutation in LDL-receptor: *Alu-Alu* recombination deletes exons encoding transmembrane and cytoplasmic domains. *Science* **227**, 140-146.

Lenardo MJ, Baltimore D. (1989) NFκB: a pleiotropic mediator of inducible and tissue-specific gene control. *Cell* **58**, 227-229.

Lenardo MJ, Pierce JW, Baltimore D. (1987) Protein binding sites in Ig gene enhancers determine transcriptional activity and inducibility. *Science* **236**, 1573-1577.

Leung K, Nabel GJ. (1988) HTLV-1 trans activator induces IL2R expression through an NFκB-like factor. *Nature* **333**, 776-778.

Levin M, Franklin EC, Frangione B, Pras M. (1972) The amino acid sequence of a major nonimmunoglobulin component of some amyloid fibrils. *J. Clin. Invest.* **51**, 2773-2776.

Levin M, Pras M, Franklin EC. (1973) Immunologic studies of the major nonimmunoglobulin protein of amyloid. I. Identification and partial characterization of a related serum component. *J. Exp. Med.* **138**, 373-380.

Levine M, Manley JL. (1989) Transcriptional repression of eukaryotic promoters. *Cell* **59**, 405-408.

Levy DE, Lew DJ, Decker T, Kessler DS, Darnell JE Jr. (1990) Synergistic interaction between IFNα and IFNγ through induced synthesis of one subunit of the transcription factor ISGF3. *EMBO J.* **9**, 1105-1111.

Lewin B. (1990) Commitment and activation at polII promoters: a tail of protein-protein interactions. *Cell* **61**, 1161-1164.

Li JJ, McAdam KPWJ. (1984) Human amyloid P component: an elastase inhibitor. *Scand. J. Immunol.* **20**, 219-226.

Li R, Knight JD, Jackson SP, Tjian R, Botchan MR. (1991) Direct interaction between Sp1 and the BPV enhancer E2 protein mediates synergistic activation of transcription. *Cell* **65**, 493-505.

Li X, Huang JH, Rienhoff HY Jr, Liao WSL. (1990) Two adjacent C/EBP binding sequences that participate in the cell specific expression of the mouse SAA3 gene. *Mol. Cell. Biol.* **10**, 6624-6631.

Liao WSL, Ma K-T, Woodworth CD, Mengel L, Isom HC. (1989) Stimulation of the acute phase response in SV40-hepatocyte cell lines. *Mol Cell. Biol.* **9**, 2779-2786.

Libermann TA, Baltimore D. (1990) Activation of IL6 gene expression through the NFκB transcription factor. *Mol. Cell. Biol.* **10**, 561-568.

Lin J-X, Vilcek J. (1987) TNF and IL1 cause a rapid and transient stimulation of c-*fos* and c-*myc* mRNA levels in human fibroblasts. *J. Biol. Chem.* **262**, 11908-11911.

Lin Y-S, Carey M, Ptashne M, Green MR. (1990) How different eukaryotic transcriptional activators can cooperate promiscuously. *Nature* **345**, 359-361.

Loetscher H, Pan Y-CE, Lahm H-W, Gentz R, Brockhaus M, Tabuchi H, Lesslauer W. (1990) Molecular cloning and expression of the human 55kd TNF receptor. *Cell* **61**, 351-359.

Lomedico PT, Gubler U, Hellman CP, Dukovich M, Giri JG, Pan YE, Collier K, Seminow R, Chua AO, Mizel SB. (1984) Cloning and expression of murine IL1 cDNA in *E. coli*. *Nature* **312**, 458-462.

Lowell CA, Potter DA, Stearman RS, Morrow JF. (1986a) Structure of the murine serum amyloid A gene family: gene conversion. *J. Biol. Chem.* **261**, 8442-8452.

Lowell CA, Stearman CA, Morrow JF. (1986b) Transcriptional regulation of serum amyloid A gene expression. *J. Biol. Chem.* **261**, 8453-8461.

Lowenthal JW, Ballard DW, Bohnlein E, Greene WC. (1989) TNFα induces proteins that bind specifically to κB-like enhancer elements and regulate IL2Rα-chain gene expression in primary human T lymphocytes. *Proc. Natl. Acad. Sci. USA* **86**, 2331-2335.

Lowenthal JW, MacDonald HR. (1986) Binding and internalization of IL1 by T cells. Direct evidence for high- and low-affinity classes of IL1-R. *J. Exp. Med.* **164**, 1060-1074.

Lu L, Walker D, Graham CD, Waheed A, Shadduck RK, Broxmeyer HE. (1988) Enhancement of release from MHC class II antigen-positive monocytes of hematopoietic colony stimulating factors CSF-1 and G-CSF by recombinant human TNFα: synergism with rhIFNγ. *Blood* **72**, 34-41.

Lubsen NH, Aarts HJM, Scoenmakers JGG. (1988) The evolution of lenticular proteins: the ß- and γ-crystallin supergene family. *Prog. Biophys. molec. Biol.* **51**, 47-76.

Ma J, Ptashne M. (1988) Converting a eukaryotic transcriptional inhibitor into an activator. *Cell* **55**, 443-446.

McAdam KPWJ, Elin RJ, Sipe JD, Wolff SM. (1978) Changes in human SAA and CRP after etiocholanolone-induced inflammation. *J. Clin. Invest.* **61**, 390-394.

McAdam KPWJ, Li J, Knowles J, Foss NT, Dinarello CA, Rosenwasser LJ, Selinger MJ, Kaplan MM, Goodman R, Herbert PN, Bausserman LL, Nadler LM. (1982) The biology of SAA: identification of the inducer, *in vitro* synthesis, and heterogeneity demonstrated with monoclonal antibodies. *Ann. N. Y. Acad. Sci.* **389**, 126-136.

Macchia G, Baldari CT, Massone A, Telford JL. (1990) A role for protein kinase C activity in IL1 induction of IL2 gene expression but not in IL1 signal transduction. *Mol. Cell. Biol.* **10**, 2731-2737.

McCarrey JR. (1990) Molecular evolution of the human phosphoglycerokinase-2 retroposon. *Nucl. Acids Research* **18**, 949-955.

McCarty M. (1947) The occurrence during acute infections of a protein not normally present in the blood. *J. Exp. Med.* **85**, 491-498.

McCormack WT, Thompson CB. (1990) Chicken IgL variable region gene conversions display pseudogene donor preference and 5' to 3' polarity. *Genes Dev.* **4**, 548-558.

McCubbin WD, Kay CM, Narindrasorasak S, Kisilevsky R. (1988) Circular-dichroism studies on two murine serum amyloid A proteins. *Biochem. J.* **256**, 775-783.

McEvoy SM, Maeda N. (1988) Complex events in the evolution of the haptoglobin gene cluster in primates. *J. Biol. Chem.* **263**, 15740-15747.

Macintyre SS, Kushner I, Samols D. (1985) Secretion of CRP becomes more efficient during the course of the acute phase response. *J. Biol. Chem.* **260**, 4169-4173.

Mackiewicz A, Ganapathi MK, Schultz D, Kushner I. (1987) Monokines regulate the glycosylation of acute phase proteins. *J. Exp. Med.* **166**, 253-258.

Mader S, Kumar V, de Verneuil H, Chambon P. (1989) Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid response element. *Nature* **338**, 271-274.

Magielska-Zero D, Rokita H, Cleszka K, Kurdowska A, Koj A, Sipe JD, Gauldie J. (1987) Comparison of the acute phase response of cultured Morris hepatoma 7777 cells and of rat hepatocytes. *Br. J. exp. Path.* **68**, 485-492.

Magielska-Zero D, Bereta J, Czuba-Pelech B, Pajolak W, Gauldie J, Koj A. (1988) Inhibitory effect of human recombinant IFNγ on synthesis of acute phase proteins in human hepatoma HepG2 cells stimulated by lymphocyte cytokines, TNFα, and IFNß$_2$/BSF-2/IL-6. *Biochem. Int.* **17**, 17-23.

Majello B, Arcone R, Toniatti C, Ciliberto G. (1990) Constitutive and IL6-induced nuclear factors that interact with the human CRP promoter. *EMBO J.* **9**, 457-465.

Maliszewski CR, Sato TA, Vanden Bos T, Waugh S, Dower SK, Slack J, Beckmann MP, Grabstein KH. (1990) Cytokine receptors and B cell functions: I. recombinant soluble receptors specifically inhibit IL1- and IL4-induced B cell activities *in vitro*. *J. Immunol.* **144**, 3028-3033.

Malter JS. (1989) Identification of AUUUA-specific mRNA binding protein. *Science* **246**, 664-666.

Manak JR, de Bisschop N, Kris RM, Prywes R. (1990) CKII enhances the binding of SRF. *Genes Dev.* **4**, 955-967.

Mangelsdorf DJ, Ong ES, Dyck JA, Evans RM. (1990) Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* **345**, 224-229.

Maniatis T, Fritsch EF, Sambrook J. (1982) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory.

Maniatis T, Goodbourn S, Fischer JA. (1987) Regulation of inducible and tissue-specific gene expression. *Science* **236**, 1237-1245.

Mann KG, Jenny RJ, Krisnaswamy S. (1988) Cofactor proteins in the assembly and expression of blood clotting enzyme complexes. *Annu. Rev. Biochem.* **57**, 915-956.

March CJ, Mosley B, Larsen A, Cerrretti DP, Braedt G, Price V, Gillis S, Henney CS, Kronheim SR, Grabstein K, Conlon PJ, Hopp TP, Cosman D. (1985) Cloning, sequence and expression of two distinct human IL1 cDNAs. *Nature* **315**, 641-647.

Marhaug G, Husby G. (1981) Characterization of human amyloid-related protein SAA as a polymorphic protein: association with albumin and prealbumin. *Clin. Exp. Immunol.* **45**, 97-106.

Marhaug G, Husby G, Dowton SB. (1990) Mink serum amyloid A protein: expression and primary structure based on cDNA sequence. *J. Biol. Chem.* **265**, 10049-10054.

Marinkovic S, Baumann H. (1990) Structure, hormonal regulation and identification of the IL6 and dexamethasone response element of the rat haptoglobin gene. *Mol. Cell. Biol.* **10**, 1573-1583.

Marinkovic S, Jahreis GP, Wong, GG, Baumann H. (1989) IL6 modulates the synthesis of a specific set of acute phase plasma proteins *in vivo*. *J. Immunol.* **142**, 808-812.

Martin KJ, Lillie JW, Green MR. (1990) Evidence for interaction of different eukaryotic transcriptional activators with distinct cellular targets. *Nature* **346**, 147-152.

Matsushima K, Akahoshi T, Yamada M, Furutani Y, Oppenheim JJ. (1986) Properties of a specific IL1-R on human EBV-transformed B lymphocytes: identity of the receptor for IL1α and IL1ß. *J. Immunol.* **136**, 4496-4502.

Maxam AM, Glbert W. (1980) Sequencing end-labelled DNA with base-specific chemical cleavages. *Methods Enzymol.* **65**, 499-560.

May LT, Ghrayeb J, Santhanam U, Tatter SB, Sthoeger Z, Helfgott DC, Chiorazzi N, Greininger G, Sehgal PB. (1988) Synthesis and secretion of multiple forms of β_2IFN/BCDF2/HSF by human fibroblasts and monocytes. *J. Biol. Chem.* **263**, 7760-7766.

Meek RL, Benditt EP. (1986) Amyloid A gene family expression in different mouse tissues. *J. Exp. Med.* **164**, 2006-2017.

Meek RL, Benditt EP. (1989a) Expression of the third member of the serum amyloid A gene family in mouse adipocytes. *J. Exp. Med.* **169**, 1841-1846.

Meek RL, Benditt EP. (1989b) Rat tissues express serum amyloid A protein-related mRNAs. *Proc. Natl. Acad. Sci. USA* **86**, 1890-1894.

Meek RL, Eriksen N, Benditt EP. (1989) Serum amyloid A in the mouse: sites of uptake and mRNA expression. *Am. J. Pathol.* **135**, 411-419.

Meek RL, Hoffman JS, Benditt EP. (1986) Amyloidogenesis: one serum amyloid A isotype is selectively removed from the circulation. *J. Exp. Med.* **163**, 499-510.

Merritt CM, Easteal S, Board PG. (1990) Evolution of human α_1-acid glycoprotein genes and surrounding *Alu* repeats. *Genomics* **6**, 659-665.

Messing J. (1983) New M13 vectors for cloning. *Methods Enzymol.* **101**, 20-78.

Meyer R, Hatada EN, Hohmann H-P, Haiker M, Bartsch C, Rothlisberger U, Lahm H-W, Schlaeger EJ, van Loon APGN, Scheidereit C. (1991) Cloning of the DNA-binding subunit of human nuclear factor κB: The level of its mRNA is strongly regulated by phorbol ester and TNFα. *Proc. Natl. Acad. Sci. USA* **88**, 966-970.

Miksicek R, Heber A, Schmid W, Danesch U, Posseckert G, Beato M, Schutz G. (1986) Glucocortcoid responsiveness of the transcriptional enhancer of Moloney murine sarcoma virus. *Cell* **46**, 283-290.

Mirkovitch J, Darnell JE Jr. (1991) Rapid in vivo footprinting technique identifies proteins bound to the TTR gene in mouse liver. *Genes Dev.* **5**, 83-93.

Mitchell PJ, Tjian R. (1989) Transcriptional regulation in mammalian cells by sequence specific DNA binding proteins. *Science* **245**, 371-378.

Mitchell TI, Coon CI, Brinckerhoff CE. (1991) Serum amyloid A (SAA3) produced by rabbit synovial fibroblasts treated with phorbol esters or interleukin 1 induces synthesis of collagenase and is neutralized with specific antiserum. *J. Clin. Invest.* **87**, 1177-1185.

Miura N, Prentice HL, Scneider PM, Perlmutter DH. (1987) Synthesis and regulation of the 2 human complement C4 genes in stable transfected mouse fibroblasts. *J. Biol. Chem.* **262**, 7928-7305.

Mizel SB. (1989) The interleukins. *FASEB J.* **3**, 2379-2388.

Mizel SB. (1990) Cyclic AMP and interleukin 1 signal transduction. *Immunol. Today* **11**, 390-391.

Mizel SB, Kilian PL, Lewis JC, Paganelli KA, Chizzonite RA. (1987) The interleukin 1 receptor. Dynamics of IL1 binding and internalization in T cells and fibroblasts. *J. Immunol.* **138**, 2906-2912.

Mizutani H, May LT, Sehgal PB, Kupper TS. (1989) Synergistic interactions of IL1 and IL6 in T cell activation. *J. Immunol.* **143**, 896-901.

Molitor JA, Walker WH, Doerre S, Ballard DW, Greene WC. (1990) NFκB: a family of inducible and differentially expressed enhancer-binding proteins in T cells *Proc. Natl. Acad. Sci. USA* **87**, 10028-10032.

Monaci P, Nicosia A, Cortese R. (1988) Two different liver-specific factors stimulate *in vitro* transcription from the human α_1AT promoter. *EMBO J.* **7**, 2075-2087.

Monisaki T, Sabina RL, Holmes EW. (1990) Adenylate deaminase: a multigene family in humans and rats. *J. Biol. Chem.* **265**, 11482-11486.

Morrone G, Ciliberto G, Oliviero S, Arcone R, Dente L, Content J, Cortese R. (1988) Recombinant interleukin 6 regulates the transcriptional activation of a set of human acute phase genes. *J. Biol. Chem.* **263**, 12554-12558.

Morrone G, Cortese R, Sorrentino V. (1989) Post-transcriptional control of negative acute phase protein genes by transforming growth factor beta. *EMBO J.* **8**, 3767-3771.

Morrow JF, Stearman RS, Peltzman CG, Potter DA. (1981) Induction of hepatic synthesis of serum amyloid A protein and actin. *Proc. Natl. Acad. Sci. USA* **78**, 4718-4722.

Mortensen RF, Beisel K, Zeleznik NJ, Le PT. (1983) Acute phase reactants of mice: II. Strain dependence of SAP levels and response to inflammation. *J. Immunol.* **130**, 885-889.

Mortensen RF, Shapiro J, Lin B-F, Douches S, Neta R. (1988) Interaction of rIL1 and rTNF in the induction of mouse acute phase proteins. *J. Immunol.* **140**, 2260-2266.

Moshage HJ, Roelofs HMJ, van Pelt JF, Hazenburg BPC, van Leeuwen MA, Limburg PC, Aarden LA, Yap SH. (1988) The effect of interleukin-1, interleukin-6 and its interrelationship on the synthesis of serum amyloid A and C-reactive protein in primary cultures of adult human hepatocytes. *Biocem. Biophys. Res. Comm.* **155**, 112-117.

Mossalayi MD, Lecron J-C, Dalloul AH, Sarfati M, Bertho J-M, Hofstetter H, Delespesse G, Debre P. (1990) Soluble CD23 and IL1 synergistically induce early human thymocyte proliferation. *J. Exp. Med.* **171**, 959-964.

Mount SM. (1982) A catalogue of splice junction sequences. *Nucl. Acids Research* **10**, 459-472.

Muegge K, Williams TM, Kant J, Karin M, Chiu R, Schmidt A, Siebenlist U, Young HA, Durum SK. (1990) IL1 costimulatory activity on the IL2 promoter via AP-1. *Science* **246**, 249-251.

Mueller CR, Maire P, Schibler U. (1990) DBP, a liver-enriched transcriptional activator, is expressed late in ontogeny and its tissue specificity is determined posttranscriptionally. *Cell* **61**, 279-291.

Mukaida N, Mahe Y, Matsushima K. (1990) Cooperative interaction of NFκB- and C/EBP-like factor binding elements in activating the IL-8 gene by pro-inflammatory cytokines. *J. Biol. Chem.* **265**, 21128-21133.

Munoz E, Beutner U, Zubiaga A, Huber BT. (1990) IL1 activates two separate signal transduction pathways in T helper type II cells. *J. Immunol.* **144**, 964-969.

Murre C, McCaw PS, Baltimore D. (1989a) A new DNA binding domain and dimerization motif in Ig enhancer binding, *daughterless*, *MyoD*, and *myc* proteins. *Cell* **56**, 777-783.

Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, Cabrera CV, Buskin JN, Hauschka SD, Lassar AB, Weintraub H, Baltimore D. (1989b) Interactions between heterologous HLH proteins generate complexes that bind specifically to a common DNA sequence. *Cell* **58**, 537-544.

Nabel GJ, Baltimore D. (1987) An inducible transcription factor activates expression of HIV in T cells. *Nature* **326**, 711-713.

Naitoh Y, Fukata J, Tominaga T, Nakai Y, Tamai S, Mori K, Imura H. (1988) IL6 stimulates the secretion of ACTH in conscious, freely moving rats. *Biochem Biophys. Res. Comm.* **155**, 1459-1463.

Nakajima K, Wall R. (1991) IL6 signals activating *junB* and TIS11 gene transcription in a B-cell hybridoma. *Mol. Cell. Biol.* **11**, 1409-1418.

Nakamura T, Donovan DM, Hamada K, Sax CM, Norman B, Flanagan JR, Ozato K, Westphal H, Piatagorski J. (1990) Regulation of the mouse αA-crystallin gene: isolation of a cDNA encoding a protein

that binds to a *cis* sequence motif shared with the MHC class I gene and other genes. *Mol. Cell. Biol.* **10**, 3700-3708.

Narkates AJ, Volanakis JE. (1982) CRP binding specificities: artificial and natural phospholipid bilayers. *Ann. N. Y. Acad. Sci* **389**, 172-182.

Nawroth PP, Bank I, Handley D, Cassimeris J, Chess L, Stern D. (1986) TNF/cachectin interacts with endothelial cell receptors to induce release of IL1. *J. Exp. Med.* **163**, 1363-1375.

Nelsen B, Hellman L, Sen R. (1988) The NFκB binding site mediates phorbol ester-inducible transcription in non lymphoid cells. *Mol. Cell. Biol.* **8**, 3526-3531.

Nicholls RD, Fischel-Ghodsian N, Higgs DR. (1987) Recombination at the human α-globin gene cluster: sequence features and topological constraints. *Cell* **49**, 369-378.

Nicosia A, Monaci P, Tomei L, De Francesco R, Nuzzo M, Stunnenburg H, Cortese R. (1990) A myosin-like dimerization helix and an extra-large homeodomain are essential elements of the tripartite DNA binding structure of LFB1. *Cell* **61**, 1225-1236.

Nishida T, Takano M, Kawakami T, Nishino N, Nakai S, Hirai Y. (1988) The transcription of the IL1ß gene is induced with PMA and inhibited with dexamethasone in U937 cells. *Biochem. Biophys Res. Comm.* **156**, 269-274.

Nishizawa M, Nagata S. (1990) Regulatory elements responsible for inducible expression of GC-SF gene in macrophages. *Mol. Cell Biol.* **10**, 2002-2011.

Nolan GP, Ghosh S, Liou H-C, Tempst P, Baltimore D. (1991) DNA binding and IκB inhibition of the cloned p65 subunit of NFκB, a *rel*-related polypeptide. *Cell* **64**, 961-969.

Nonaka M, Gitlin JD, Colten HR. (1989) Regulation of human and murine complement: comparison of 5' structure and functional elements regualting human and murine factor B gene expression. *Mol. Cell. Biochem.* **89**, 1-14.

Nonaka M, Huang Z-M. (1990) IL1-mediated enhancement of mouse factor B gene expression via NFκB-like hepatoma nuclear factor. *Mol. Cell. Biol.* **10**, 6283-6289.

Nophar Y, Kemper O, Brakebusch C, Engelmann H, Zwang R, Aderka D, Holtmann H, Wallach D. (1990) Soluble forms of TNF-receptors (TNF-Rs). The cDNA for the type I TNF-R, cloned using amino acid sequence data of its soluble form, encodes both the cell surface and a soluble form of the receptor. *EMBO J.* **9**, 3269-3278.

Nordan RP, Pumphrey JG, Rudikoff S. (1987) Purification and NH_2-terminal sequence of a plasmacytoma growth factor derived from the murine macrophage cell line P388D1. *J. Immunol.* **139**, 813-817.

Ohno S. (1984) Repeats of base oligomers as the primordial coding sequences of the primeval earth and their vestiges in modern genes. *J. Mol. Evol.* **20**, 313-321.

Ohta T. (1988) Time for acquiring a new gene by duplication. *Proc. Natl. Acad. Sci. USA* **85**, 3509-3512.

Old LJ. (1988) Tumor necrosis factor. *Sci. Am.* **258**, 59-75.

Oliviero S, Cortese R. (1989) The human haptoglobin gene promoter: IL6REs interact with a DNA binding protein induced by IL6. *EMBO J.* **8**, 1145-1151.

Oliviero S, Morrone G, Cortese R. (1987) The human haptoglobin gene: transcriptional regulation during development and AP induction. *EMBO J.* **6**, 1905-1912.

O'Neill LAJ, Bird TA, Saklatvala J. (1990a) Interleukin 1 signal transduction. *Immunol. Today* **11**, 392-394.

O'Neill LAJ, Bird TA, Gearing AJH, Saklatvala J. (1990b) IL-1 signal transduction. Increased GTP binding and hydrolysis in membranes of a murine thymoma cell line (EL4). *J. Biol. Chem.* **265**, 3146-3152.

Oppenheim JJ, Kovacs EJ, Matsushima K, Durum SK. (1986) There is more than one interleukin 1. *Immunol. Today* 7, 45-56.

Osborn L, Kunkel S, Nabel GJ. (1989) TNF and IL1 stimulate the HIV enhancer by activation of NFκB. *Proc. Natl. Acad. Sci. USA* **86**, 2336-2340.

Ostrowski J, Meier KE, Stanton TH, Smith LL, Bomsztyk K. (1988) IFNγ and IL1α induce transient translocation of protein kinase C activity to membranes in a B lymphoid cell line. Evidence for a PKC-independent pathway in lymphokine-induced cytoplasmic alkalinization. *J. Biol. Chem.* **263**, 13786-13790.

Paonessa G, Gounari F, Frank R, Cortese R. (1988) Purification of a NF1-like DNA binding protein from rat liver and cloning of the corresponding DNA. *EMBO J.* 7, 3115-3123.

Parmelee DC, Titani K, Ericsson LH, Eriksen N, Benditt EP, Walsh KA. (1982) Amino acid sequence of amyloid-related apoprotein (apoSAA$_1$) from human high-density lipoprotein. *Biochemistry* **21**, 3298-3303.

Pennica D, Nedwin GE, Hayflick JS, Seeburg PH, Derynck R, Palladino MA, Kohr WJ, Aggarwal BB, Goeddel DV. (1984) Human TNF: precursor structure, expression and homology to lymphotoxin. *Nature* **312**, 724-729.

Pepys MB, Baltz ML. (1983) Acute phase proteins with special reference to CRP and related proteins (pentraxins) and SAA protein. *Adv. Immunol.* 34, 141-211.

Pepys MB, Butler PJG. (1987) SAP component is the major calcium-dependent specific DNA binding protein of the serum. *Biochem. Biophys. Res. Comm.* **148**, 308-313.

Pepys MB, Dash AC, Munn EA, Feinstein A, Skinner M, Cohen AS, Gewurz H, Osmand AP, Painter RH. (1977) Isolation of amyloid P component (protein AP) from normal serum as a calcium-dependent binding protein. *Lancet* **1**, 1029-1031.

Perez C, Albert I, DeFay K, Zachariades N, Gooding L, Kriegler M. (1990) A nonsecretable cell surface mutant of TNF kills by cell-to-cell contact. *Cell* **63**, 251-258.

Peristeris P, Gaspar A, Gros P, Laurent P, Bernon H, Bienvenu J. (1989) Effects of serum amyloid A protein on lymphocytes, HeLa, and MRC$_5$ cells in culture. *Biochem. Cell. Biol.* **67**, 365-370.

Perlmutter DH, Dinarello CA, Punsal PI, Colten HR. (1986a) Cachectin/tumour necrosis factor regulates hepatic acute-phase gene expression. *J. Clin. Invest.* **78**, 1349-1354.

Perlmutter DH, Goldberger G, Dinarello CA, Mizel SB, Colten HR. (1986b) Regulation of class III major histocompatibility complex gene products by interleukin-1. *Science* **232**, 850-852.

Perlmutter DH, Strunk RC, Goldberger G, Cole FS. (1986c) Regulation of complement proteins C2 and factor B by interleukin-1 and interferon-gamma acting on transfected L cells. *Mol. Immunol.* **23**, 1263-1266.

Pessara U, Koch N. (1990) TNFα regulates expression of the MHC class II-associated invariant chain by binding of an NFκB-like factor to a promoter element. *Mol. Cell. Biol.* **10**, 4146-4154.

Peterson MG, Hannan F, Mercier JFB. (1988) The sheep metallothionein gene family. *Eur. J. Biochem.* **184**, 417-424.

Pettersson M, Schaffner W. (1990) Synergistic activation of transcription by multiple binding sites for NFκB even in the absence of cooperative binding to DNA. *J. Mol. Biol.* **214**, 373-380.

Pfeilschifter J, Schwarzenbach H. (1990) IL1 and TNF stimulate cGMP formation in rat renal mesangial cells. *FEBS Lett.* **273**, 185-187.

Pober JS, Gimbrone MA Jr, Lapierre LA, Mendrick DL, Fiers W, Rothlein R, Springer TA. (1986) Overlapping patterns of activation of human endothelial cells by IL1, TNF and immune IFN. *J. Immunol.* **137**, 1893-1896.

Poli V, Cortese R. (1989) IL6 induces a liver-specific nuclear protein that binds to the promoter of acute phase genes. *Proc. Natl. Acad. Sci. USA* **86**, 8202-8206.

Poli V, Mancini FP, Cortese R. (1990) IL6DBP, a nuclear protein involved in IL6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. *Cell* **63**, 643-653.

Poli V, Silengo L, Altruda F, Cortese R. (1989) The analysis of the human hemopexin promoter defines a new class of liver-specific genes. *Nucl. Acids Research* **17**, 9351-9365.

Ponglikitmongkol M, White JH, Chambon P. (1990) Synergistic activation of transcription by the human estrogen receptor bound to tandem responsive elements. *EMBO J.* **9**, 2221-2231.

Prowse KR, Baumann H. (1988) HSF, ß$_2$IFN, and IL1 enhance expression of the rat α_1AGP gene via a distal upstream regulatory region. *Mol. Cell. Biol.* **8**, 42-51.

Ptashne M. (1988) How eukaryotic transcriptional activators work. *Nature* **335**, 683-689.

Ptashne M, Gann AF (1990) Activators and targets. *Nature* **345**, 783-786.

Pugh BF, Tjian R. (1990) Mechanism of transcriptional activation by Sp1: evidence for coactivators. *Cell* **61**, 1187-1197.

Qwarnstrom EE, Page RC, Gillis S, Dower SK. (1988) Binding, internalization, and intracellular localization of IL1ß in human diploid fibroblasts. *J. Biol. Chem.* **263**, 8261-8269.

Ramadori G, Sipe JD, Colten HR. (1985a) Expression and regulation of the murine serum amyloid A (SAA) gene in extrahepatic sites. *J. Immunol.* **135**, 3645-3647.

Ramadori G, Sipe JD, Dinarello CA, Mizel SB, Colten HR. (1985b) Pretranslational modulation of acute phase hepatic protein synthesis by murine recombinant interleukin 1 (IL-1) and purified human IL-1. *J. Exp. Med.* **162**, 930-942.

Rangan VS, Das GC. (1990) Purification and biochemical characterization of hepatocyte nuclear factor 2 involved in liver-specific transcription of the human α_1-antitrypsin gene. *J. Biol. Chem.* **265**, 8874-8879.

Ray A, LaForge KS, Sehgal PB. (1990) On the mechanism for efficient repression of the IL6 promoter by glucocorticoids: enhancer, TATA box and RNA start site occlusion. *Mol. Cell. Biol.* **10**, 5736-5746.

Raynal M-C, Lin Z, Hirano T, Mayer L, Kishimoto T, Chen-Kiang S. (1989) IL6 induces secretion of IgG1 by coordinated transcriptional activation and differential mRNA accumulation. *Proc. Natl. Acad. Sci. USA* **86**, 8024-8028.

Reinke R, Feigelson P. (1985) Rat α_1-acid glycoprotein. Gene sequence and regulation by glucocorticoids in transfected L cells. *J. Biol. Chem.* **260**, 4397-4403.

Renkawitz R. (1990) Transcriptional repression in eukaryotes. *Trends. Genet.* **6**, 192-196.

Renkonen R, Mattila P, Hayry P, Ustinov J. (1990) IL1-induced lymphocyte binding to endothelial cells. Role of cAMP as a second messenger. *Eur. J. Immunol.* **20**, 1563-1567.

Rheaume C, Latimer JJ, Baumann H, Berger FG. (1988) Tissue- and species-specific regulation of murine α_1-antitrypsin gene transcription. *J. Biol. Chem.* **263**, 15118-15121.

Rienhoff HY Jr. (1989) Identification of a transcriptional enhancer in a mouse amyloid gene. *J. Biol. Chem.* **264**, 419-425.

Rienhoff HY Jr, Groudine M. (1988) Regulation of amyloid A gene expression in cultured cells. *Mol. Cell. Biol.* **8**, 3710-3716.

Rienhoff HY Jr, Huang JH, Li X, Liao WSL. (1990) Molecular and cellular biology of serum amyloid A. *Mol. Biol. Med.* **7**, 287-298.

Rigby PWJ, Dieckmann M, Rhodes C, Berg P. (1977) Labelling DNA to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* **113**, 237-251.

Ritchie DG, Levy BA, Adams MA, Fuller GM (1982) Regulation of fibrinogen synthesis by plasmin-derived fragments of fibrinogen and fibrin: an indirect feedback pathway. *Proc. Natl. Acad. Sci. USA* **79**, 1530-1534.

Robertson M. (1988) Homoeoboxes, POU proteins and the limits to promiscuity. *Nature* **336**, 522-524.

Robey FA, Jones KD, Steinberg AD. (1985) CRP mediates the solubilization of nuclear DNA by complement *in vitro*. *J. Exp. Med.* **161**, 1344-1356.

Robey FA, Liu T-Y. (1981) Limulin: a CRP from *Limulus polyphemus*. *J. Biol. Chem.* **256**, 969-975.

Robey FA, Ohura K, Futaki S, Fujii N, Yajima H, Goldmann N, Jones KD, Wahl S. (1987) Proteolysis of human CRP produces peptides with potent immunomodualting activity. *J. Biol. Chem.* **262**, 7053-7057.

Rodan SB, Wesolowski G, Chin J, Limjuco GA, Schmidt JA, Rodan GA. (1990) IL1 binds to high affinity receptors on human osteosarcoma cells and potentiates PGE2 stimulation of cAMP production. *J. Immunol.* **145**, 1231-1237.

Rokita H, Shirahama T, Cohen AS, Meek RL, Benditt EP, Sipe JD. (1987) Differential expression of the amyloid SAA3 gene in the liver and peritoneal macrophages of mice under going dissimilar inflammatory episodes. *J. Immunol.* **139**, 3849-3853.

Roman C, Platero JS, Shuman J, Calame K. (1990) Ig/EBP1: a ubiquitously expressed Ig enhancer binding protein that is similar to C/EBP and heterodimerizes with C/EBP. *Genes Dev.* **4**, 1404-1415.

Ron D, Brasier AR, Habener JF. (1991) Angiotensinogen gene-inducible enhancer binding protein 1, a member of a new family of large nuclear proteins that recognize NFκB-binding sites through a zinc finger motif. *Mol. Cell. Biol.* **11**, 2887-2895.

Ron D, Brasier AR, Wright KA, Tate JE, Habener JF. (1990) An inducible 50kD NFκB-like protein and a constitutive protein both bind the APRE of the angiotensinogen gene. *Mol. Cell. Biol.* **10**, 1023-1032.

Rose-John S, Hipp E, Lenz D, Legres LG, Korr H, Hirano T, Kishimoto T, Heinrich PC. (1991) Structural and functional studies on the human IL6 receptor. *J. Biol. Chem* **266**, 3841-3846.

Rose-John S, Schooltink H, Lenz D, Hipp E, Dufhues G, Schmitz, H, Schiel X, Hirano T, Kishimoto T, Heinrich PC. (1990) Studies on the structure and regulation of the human hepatic IL6 receptor. *Eur. J. Biochem.* **190**, 79-83.

Rosenthal CJ, Franklin EC. (1975) Variation with age and disease of an amyloid A protein-related serum component. *J. Clin. Invest.* **55**, 746-753.

Rosoff PM, Savage N, Dinarello CA. (1988) IL1 stimulates diacylglycerol production in T lymphocytes by a novel mechanism. *Cell* **54**, 73-81.

Roy C, Lebleu B. (1990) DNA-protein interactions at the IFN-responsive promoter: evidences for an involvement of phosphorylation. *Nucl. Acids Res.* **18**, 2125-2137.

Rubartelli A, Cozzolino F, Talio M, Sitia R. (1990) A novel secretory pathway for IL1ß, a protein lacking a signal sequence. *EMBO J.* **9**, 1503-1510.

Ruben SM, Dillon PJ, Schreck R, Henkel T, Chen C-H, Maher M, Baeuerle PA, Rosen CA. (1991) Isolation of a *rel*-related human cDNA that potentially encodes the 65kD subunit of NFκB. *Science* **251**, 1490-1493.

Ruggiero V, Tavernier J, Fiers W, Baglioni C. (1986) Induction of the synthesis of TNF-R by IFNγ. *J. Immunol.* **136**, 2445-2450.

Rustgi AK, Van't Veer LJ, Bernards R. (1990) Two genes encode factors with NFκB and H2TF1-like DNA binding properties. *Proc. Natl. Acad. Sci. USA* **87**, 8707-8710.

Sack GH Jr. (1983) Molecular cloning of human genes for serum amyloid A. *Gene*, **21**, 19-24.

Sack GH Jr. (1988) Serum amyloid A (SAA) gene variations in familial Mediterranean fever. *Mol. Biol. Med.* **5**, 61-67.

Sack GH Jr, Talbot CC Jr. (1989) The human serum amyloid A (SAA)-encoding gene *GSAA1*; nucleotide sequence and possible autocrine collagenase inducer function. *Gene* **84**, 509-515.

Sack GH Jr, Talbot CC Jr, Seuanez H, O'Brien SJ. (1989) Molecular analysis of the human serum amyloid A (SAA) gene family. *Scand. J. Immunol.* **29**, 113-119.

Sanger F, Nicklen S, Coulson AR. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.

Sapolsky R, Rivier C, Yamamoto G, Plotsky P, Cale W. (1987) IL1 stimulates the secretion of hypothalamic corticotropin-releasing factor. *Science* **238**, 522-524.

Satoh T, Nakamura S, Taga T, Matsuda T, Hirano T, Kishimoto T, Kaziro Y. (1988) Induction of neural differentiation in PC12 cells by BSF2/IL6. *Mol. Cell. Biol.* **8**, 3546-

Schall TJ, Lewis M, Koller KJ, Lee A, Rice GC, Wong GHW, Gatanaga T, Granger GA, Lentz R, Raab H, Kohr WJ, Goeddel DV. (1990) Molecular cloning of a receptor for human TNF. *Cell* **61**, 361-370.

Schatt MD, Rusconi S, Schaffner W. (1990) A single DNA binding transcription factor is sufficient for activation from a distant enhancer and from a promoter position. *EMBO J.* **9**, 481-487.

Schatz C, Chatton B. (1990) Stereoalignment requirements for activation of transcription by the SV40 enhancer. *Nucl. Acids Res.* **18**, 421-427.

Schaufele F, West BL, Reudelhuber TL. (1990) Overlapping Pit-1 and Sp1 binding sites are both essential to full rat growth hormone gene promoter activity despite mutually exclusive Pit-1 and Sp1 binding. *J. Biol. Chem.* **265**, 17189-17196.

Schimpl A, Wecker E. (1972) Replacement of T cell function by a T cell product. *Nature New Biol.* **237**, 15-17.

Schmid CW, Jelinek WK. (1982) The *Alu* family of dispersed repetitive sequences. *Science* **216**, 1065-1070.

Schmid W, Strahle U, Schutz G, Schmitt J, Stunnenberg G. (1989) Glucocorticoid receptor binds cooperatively to adjacent recognition sites. *EMBO J.* **8**, 2257-2263.

Schreck R, Baeuerle PA. (1990) NFκB as inducible activator of the GMCSF gene. *Mol. Cell Biol.* **10**, 1281-1286.

Schreiber G. (1987) Synthesis, processing, and secretion of plasma proteins by the liver and other organs and their regulation. In "The plasma proteins: structure, function and genetic control" (Putnam, F. W. ed.), Academic Press. vol. V, pp. 293-363,

Schreiber G, Howlett G, Nagashima M, Millership A, Martin H, Urban J, Kotter L. (1982) The acute phase response of plasma protein synthesis during experimental inflammation. *J. Biol. Chem.* **257**, 10271-10277.

Schreier PH, Bothwell AL, Mueller-Hill B, Baltimore D. (1981) Multiple differences between nucleic acid sequences of the IgG2aa and IgG2ab alleles of the mouse. *Proc. Natl. Acad. Sci. USA* **78**, 4495-4499.

Schroeder JJ, Cousins RJ. (1990) Interleukin 6 regulates metallothionein gene expression and zinc metabolism in hepatocyte monolayer cultures. *Proc. Natl. Acad. Sci. USA* **87**, 3137-3141.

Schugart K, Kappen C, Ruddle FH. (1989) Duplication of large genomic regions during the evolution of vertebrate homeobox genes. *Proc. Natl. Acad. Sci. USA* **86**, 7067-7071.

Schule R, Rangarajan P, Kliewer S, Ransone LJ, Bolado J, Yang N, Verma IM, Evans RM. (1990) Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. *Cell* **62**, 1217-1226.

Schumann RR, Leong SR, Flaggs GW, Gray PW, Wright SD, Mathison JC, Tobias PS, Ulevitch RJ. (1990) Structure and function of LPS binding protein. *Science* **249**, 1429-1431.

Schutze S, Nottrott S, Pfizenmaier K, Kronke M. (1990) TNF signal transduction; Cell-type-specific activation and translocation of protein kinase C. *J. Immunol.* **144**, 2604-2608.

Scott MP, Weiner AJ. (1984) Structural relationships among genes that control development: sequence homology between the *Antennapedia, Ultrabithorax and fushi tarazu* loci of *Drosophila. Proc. Natl. Acad. Sci. USA* **81**, 4115-4119.

Seelentag WK, Mermod J-J, Montesano R, Vassali P. (1987) Additive effects of IL1 and TNFα on the accumulation of the 3 granulocyte and macrophage colony stimulating factor mRNAs in human endothelial cells. *EMBO J.* **6**, 2261-2265.

Segrest JP, Pownall HJ, Jackson RL, Glenner GG, Pollock PS. (1976) Amyloid A: amphipathic helices and lipid binding. *Biochem.* **15**, 3187-3191.

Sehgal PB. (1990) Interleukin-6: molecular pathophysiology. *J. Invest. Dermatol.* **94**, 2S-6S.

Sehgal PB, Grieninger G, Tosato G. (eds.) (1989) Regulation of the acute phase and immune responses: interleukin 6. *Ann. N. Y. Acad. Sci.* vol. 557.

Selinger MJ, McAdam KPWJ, Kaplan MM, Sipe JD, Vogel SN, Rosensteich DL. (1980) Monokine-induced synthesis of SAA protein by hepatocytes. *Nature* **285**, 498-500.

Sellar GC, de Beer MC, Lelias JM, Snyder PW, Glickman LT, Felsburg PJ, Whitehead AS. (1991) Dog serum amyloid A protein. *J. Biol. Chem.* **266**, 3505-3510.

Sen R, Baltimore D. (1986) Multiple nuclear factors interact with the Ig enhancer sequences. *Cell*, 705-716.

Shannon MF, Pell LM, Lenardo MJ, Kuczek ES, Occhiodoro FS, Dunn SM, Vadas MA. (1990) A novel TNF-responsive transcription factor which recognizes a regulatory element in hemopoietic growth factor genes. *Mol. Cell. Biol.* **10**, 2950-2959.

Shaw JP, Utz PJ, Durand DB, Toole JJ, Emmel EA, Crabtree GR. (1988) Identification of a putative regulation of early T cell activation genes. *Science* **241**, 202-205.

Shiels BR, Taylor JM, Ringold GM. (1987) Modified nuclear processing of α_1-acid glycoprotein RNA during inflammation. *J. Biol. Chem.* **262**, 12826-12831.

Shimizu H, Mitomo K, Watanabe T, Okamoto S, Yamamoto K-I. (1990) Involvement of a NFκB-like transcription factor in the activation of the IL6 gene by inflammatory lymphokines. *Mol. Cell Biol.* **10**, 561-568.

Shirai T, Yamaguchi H, Ito H, Todd CW, Wallace RB. (1985) Cloning and expression in *E. Coli* of the gene for human TNF. *Nature* **313**, 803-806.

Shirakawa F, Chedid M, Suttles J, Pollok BA, Mizel SB. (1989) IL1 and cAMP induce κ immunoglobulin light-chain expression via activation of an NFκB-like DNA-binding protein. *Mol. Cell. Biol.* **9**, 959-964.

Shirakawa F, Mizel SB. (1989) *In vitro* activation and nuclear translocation of NFκB catalyzed by cyclic AMP-dependent protein kinase and protein kinase C. *Mol. Cell. Biol.* **9**, 2424-2430.

Shirakawa F, Yamashita U, Chedid M, Mizel SB. (1988) Cyclic AMP-an intracellular second messenger for IL1. *Proc. Natl. Acad. Sci. USA* **85**, 8201-8205.

Shiroo M, Kawahara E, Nakanishi I, Migita S. (1987) Specific deposition of serum amyloid A protein 2 in the mouse. *Scand. J. Immunol.* **26**, 709-716.

Shohat M, Shohat T, Rotter JI, Schlesinger M, Petersen GM, Pribyl T, Sack G, Schwabe AD, Korenberg JR. (1990) Serum amyloid A and P protein genes in familial Mediterranean fever. *Genomics* **8**, 83-89.

Sims JE, Acres RB, Grubin CE, McMahan CJ, Wignall JM, March CJ, Dower SK. (1989) Cloning the IL1-receptor from human T cells. *Proc. Natl. Acad. Sci. USA* **86**, 8946-8950.

Sims JE, March CJ, Cosman D, Widmer MB, MacDonald HR, McMahan CJ, Grubin CE, Wignall JM, Jackson JL, Call SM, Friend D, Alpert AR, Gillis S, Urdal DL, Dower SK. (1988) cDNA expression cloning of the IL1-receptor, a member of the Ig superfamily. *Science* **241**, 585-589.

Sipe JD. (1990) The acute phase response. *In* "Immunophysiology. Role of cells and cytokines in inflammation", (Oppenheim J, Sherach P. (eds.) Oxford University Press. pp. 259-273.

Sipe JD, Colten HR, Goldberger G, Edge MD, Tack BF, Cohen AS, Whitehead AS. (1985) Human serum amyloid A (SAA): biosynthesis and postsynthetic processing of preSAA and structural variants defined by complementary DNA. *Biochemistry* **24**, 2931-2936.

Sipe JD, Vogel SN, Ryan JL, McAdam KPWJ, Rosenstrich DL. (1979) Detection of a mediator derived from endotoxin-stimulated macrophages that induces the acute phase SAA response in mice. *J. Exp. Med.* **150**, 597-606.

Sipe JD, Woo P, Goldberger G, Cohen AS, Whitehead AS. (1986) Characterization of 2 distinct SAA gene products defined by their cDNAs. *In* Amyloidosis, the disease complex. Proc. IV International Amyloid Workshop (Glenner *et al.*, eds). Plenum Press, pp57-60.

Skogen B, Thorsteinsson L, Natvig JB. (1980) Degradation of protein SAA to an AA-like fragment by enzymes of monocytic origin. *Scand. J. Immunol.* **11**, 533-540.

Sletten K, Husebekk A, Husby G. (1989) The primary structure of equine serum amyloid A (SAA) protein. *Scand. J. Immunol.* **30**, 117-122.

Sletten K, Marhaug G, Husby G. (1983) The covalent structure of amyloid-related serum protein SAA from two patients with inflammatory disease. *Hoppe-Seyler's Z. Physiol. Chem.* **364**, 1039-1046.

Slightom JL, Blechl AE, Smithies O. (1981) Human fetal Gγ and Aγ globin genes: complete nucleotide sequences suggest that DNA can be exchanged between these duplicated genes. *Cell* **21**, 627-638.

Smeal T, Angel P, Meek J, Karin M. (1989) Different requirements for formation of Jun:Jun and Jun:Fos complexes. *Genes Dev.* **3**, 2091-2100.

Smith CA, Davis T, Anderson D, Solam L, Beckmann MP, Jerzy R, Dower SK, Cosman D, Goodwin RG. (1990) A receptor for TNF defines an unusual family of cellular and viral proteins. *Science* **248**, 1019-1022.

Smith GP. (1976) Evolution of repeated DNA sequences by unequal cross-over. *Science* **191**, 528-535.

Smith GR. (1987) Mechanism and control of homologous recombination in *E. coli. Ann. Rev. Genet.* **21**, 179-201.

Snyers L, De Wit L, Content J. (1990) Glucocorticoid-upregulation of high-affinity IL6 receptors on human epithelial cells. *Proc. Natl. Acad. Sci. USA* **87**, 2838-2842.

Southern EM. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**, 503-517.

Spies T, Morton CC, Nedospasov SA, Fiers W, Pious D, Strominger JL. (1986) Genes for the TNFs α and ß are linked to the human major histocompatibility complex. *Proc. Natl. Acad. Sci. USA* **83**, 8699-8702.

Sporn MB, Roberts AB, Wakefield LM, Assoian RK. (1986) TGFß: biological function and chemical structure. *Science* **233**, 532-534.

Stadnyk AW, Gauldie J. (1991) The acute phase protein response during parasitic infection. *Immunoparasitol. Today* **7**, A7-A12.

Steinkasserer A, Weiss EH, Schwaeble W, Linke RP. (1990) Heterogeneity of human serum amyloid A protein. *Biochem. J.* **268**, 187-193.

Steinmetz A, Hocke G, Saile R, Puchois P, Fruchart J-C. (1989) Influence of serum amyloid A on cholesterol esterification in human plasma. *Biochim. Biophys. Acta* **1006**, 173-178.

Stoeckle MY. (1991) Post-transcriptional regulation of *gro* α, ß, γ and IL-8 mRNAs by IL1ß. *Nucl. Acids Res.* **19**, 917-920.

Strachan AF, de Beer FC, Coetzee GA, Hoppe HC, Jeenah MS, van der Westhuyzen DR. (1986) Characteristics of apoSAA-containing HDL_3 in humans. *Prot. Biol. Fluids Proc. Colloq.* **34**, 359-362.

Strachan AF, de Beer FC, van der Westhuyzen DR, Coetzee GA. (1988) Identification of three isoform patterns of human serum amyloid A protein. *Biochem. J.* **250**, 203-207.

Strachan AF, Brandt WF, Woo P, van der Westhuyzen DR, Coetzee GA, de Beer MC, Shephard EC, de Beer FC. (1989a) Human serum amyloid A protein: the assignment of the six major isoforms to three published gene sequences and evidence for two genetic loci. *J. Biol. Chem.* **264**, 18368-18373.

Strachan AF, Shephard EG, Bellstedt DU, Coetzee GA, van der Westhuyzen DR, de Beer FC. (1989b) Human serum amyloid A protein: behaviour in aqueous and urea-containing solutions and antibody production. *Biochem. J.* **263**, 365-370.

Strieter RM, Kunkel SL, Showell HJ, Rennick DG, Phan SH, Ward PA, Marks RM. (1989) Endothelial cell gene expression of a neutrophil chemotactic factor by TNFα, LPS, and IL1ß. *Science* **243**, 1467-1469.

Stringer KF, Ingles CJ, Greenblatt J. (1990) Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. *Nature* **345**, 783-786.

Sudhof TC, Goldstein JL, Brown MS, Russell DW. (1985a) The LDL-R gene: a mosaic of exons shared with different proteins. *Science* **228**, 815-822.

Sudhof TC, Russell DW, Goldstein JL, Brown MS, Sauchez-Pescadov R, Bell GI. (1985b) Cassette of 8 exons shared by genes for LDL-R and EGF precursor. *Science* **228**, 893-895.

Suematsu S, Matsuda T, Aozasa K, Akira S, Nakano N, Ohno S, Miyazaki J-I, Yamamura K-I, Hirano T, Kishimoto T. (1989) IgG1 plasmacytosis in IL6 transgenic mice. *Proc. Natl. Acad. Sci. USA* **86**, 7547-7551.

Svedersky LP, Shepard HM, Spencer SA, Shalaby MR, Palladino MA. (1984) Augmentation of human natural cell-mediated cytotoxicity by rhIL2. *J. Immunol.* **133**, 714-718.

Symons JA, Duff GW. (1990) A soluble form of the IL1-receptor produced by a human B cell line. *FEBS Lett.* **272**, 133-136.

Szostak JW, Orr-Weaver TL, Rothstein RJ, Stuhl FW. (1983) The double-strand-break repair model for recombination. *Cell* **33**, 25-35.

Tabor S, Richardson CC. (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Natl. Acad. Sci. USA* **84**, 4767-4771.

Taga T, Hibi M, Hirata Y, Yamasaki K, Yasukawa K, Matsuda T, Hirano T, Kishimoto T (1989) IL6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell* **58**, 573-581.

Takai Y, Wong G, Clark S, Burakoff S, Herrmann S. (1988) B cell stimulatory factor 2 is involved in the differentiation of cytotoxic T lymphocytes. *J. Immunol.* **140**, 508-512.

Tape C, Kisilevsky R. (1990) ApoA-I and apoSAA half-lives during acute inflammation and amyloidogenesis. *Biochim. Biophys. Acta* **1043**, 295-300.

Tartof KD, Hobbs CA. (1988) New cloning vectors for rapid restriction mapping. *Gene* **67**, 169-182.

Tasset D, Tora L, Fromental C, Scheer S, Chambon P. (1990) Distinct classes of transcriptional activating domains function by different mechanisms. *Cell* **62**, 1177-1187.

Tautz D, Renz M. (1984) Simple sequences are ubiquitous repetitive elements of eukaryotic genomes. *Nucl. Acids Research* **12**, 4127-4138.

Taylor BA, Rowe L. (1984) Genes for SAA protein map to chromosome 7 in the mouse. *Mol. Gen. Genet.* **195**, 491-499.

Taylor DJ, Evanson JM, Wooley DE. (1990) Contrasting effects of the PKC inhibitor staurosprine on cytokine and phorbol ester stimulation of fructose 2,6-bisphosphate and prostaglandin E production by fibroblasts *in vitro*. *Biochem. J.* **269**, 573-577.

Thelen M, Rosen A, Nairn AC, Aderem A. (1990) TNFα modifies agonist-dependent responses in human neutrophils by inducing the synthesis and myristoylation of a specific PKC substrate. *Proc. Natl. Acad. Sci. USA* **87**, 5603-5607.

Tillet WS, Francis T Jr. (1930) Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus. *J. Exp. Med.* **52**, 561-571.

Tobias PS, McAdam KPWJ, Soldau K, Ulevitch RJ. (1985) Control of a LPS-binding acute phase reactant from rabbit serum. *Infect. Immun.* **50**, 73-76.

Tobias PS, McAdam KPWJ, Ulevitch RJ. (1982) Interactions of bacterial lipopolysaccharide with acute-phase rabbit serum and isolation of two forms of rabbit serum amyloid A. *J. Immunol.* **128**, 1420-1427.

Tobias PS, Soldau K, Ulevitch RJ. (1986) Isolation of a LPS-binding acute phase protein from rabbit serum. *J. Exp. Med.* **164**, 777-793.

Toniatti C, Demartis A, Monaci P, Nicosia A, Ciliberto G. (1990) Synergistic *trans*-activation of the human CRP promoter by transcription factor HNF1 binding at two distinct sites. *EMBO J.* **9**, 4467-4475.

Tracey KJ, Vlassar H, Cerami A. (1989) Cachectin/TNF. *Lancet* **1**, 1122-1125.

Travis J, Salvesen GS. (1983) Human plasma proteinase inhibitors. *Annu. Rev. Biochem.* **52**, 655-709.

Tsai SY, Tsai MJ, O'Malley BW. (1989) Cooperative binding of steroid hormone receptors contributes to transcriptional synergy at target enhancer elements. *Cell* **57**, 443-448.

Tsujimoto M, Vilcek J. (1986) TNF-Rs in HeLa cells and their regulation by IFNγ. *J. Biol. Chem.* **261**, 5384-5388.

Tsujimoto M, Yip YK, Vilcek J. (1986) IFNγ enhances expression of cellular receptors for TNF. *J. Immunol.* **136**, 2441-2444.

Turnell W, Sarra R, Glover ID, Baum JO, Caspi D, Baltz ML, Pepys MB. (1986a) Secondary structure prediction of human SAA_1 presumptive identification of calcium and lipid binding sites. *Mol. Biol. Med.* **3**, 387-407.

Turnell W, Sarra R, Baum JO, Caspi D, Baltz ML, Pepys MB. (1986b) X-ray scattering and diffraction by wet gels of AA amyloid fibrils. *Mol. Biol. Med.* **3**, 409-424.

Turner R, Tjian R. (1989) Leucine repeats and an adjacent DNA binding domain mediate the formation of functional cFos-cJun heterodimers. *Science* **243**, 1689-1694.

Van Damme J, Opdenakker G, Simpson RJ, Rubira MR, Caphas S, Vink A, Billiau A, Van Snick J. (1987) Identification of the human 26-kD protein IFNß$_2$ as a B cell hybridoma/plasmacytoma growth factor induced by IL1 and TNF. *J. Exp. Med.* **165**, 914-919.

van de Wetering M, Oosterwegel M, Dooijes D, Clevers H. (1991) Identification and cloning of TCF-1 a T lymphocyte specific transcription factor containing a sequence specific HMG box. *EMBO J.* **10**, 123-132.

van Dijk W, Pos O, van der Stelt ME, Moshage HJ, Yap SH, Dente L, Baumann P, Eap CG. (1991) Inflammation-induced changes in expression and glycosylation of genetic variants of α_1AGP. *Biochem. J.* **276**, 343-347.

Vannice JL, Taylor JM, Ringold GM. (1984) Glucocorticoid-mediated induction of α_1AGP: evidence for hormone-regulated RNA processing. *Proc. Natl. Acad. Sci. USA* **81**, 4241-4245.

Van Snick J. (1990) Interleukin 6: an overview. *Annu. Rev. Immunol.* **8**, 253-278.

Visvanathan KV, Goodbourn S. (1989) dsRNA activates binding of NFκB to an inducible element in the human ßIFN promoter. *EMBO J.* **8**, 1129-1138.

Vosatka RJ, Hermanowski-Vosatka A, Metz R, Ziff EB. (1989) Dynamic interactions of c-fos protein in serum-stimulated 3T3 cells. *J. Cell. Physiol.* **138**, 493-502.

Waalen K, Sletten K, Husby G, Nordstaga K. (1980) The primary structure of amyloid fibril protein AA in endotoxin-induced amyloidosis of the mink. *Eur. J. Biochem.* **104**, 407-412.

Wadey RB, Little PFR, Pritchard J, Cowell JK. (1990) Isolation and regional localisation of DNA sequences from a human chromosome 11-specific cosmid library. *Hum. Genet.* **84**, 417-423.

Wallace MR, Conneally PM, Benson MD. (1988) A DNA test for Indiana/Swiss hereditary amyloidosis (FAPII). *Am. J. Hum. Genet.* **43**, 182-187.

Walsh JB. (1987) Sequence-dependent gene conversion: can duplicated genes diverge fast enough to escape conversion? *Genetics* **117**, 543-557.

Walter P, Blobel G. (1982) SRP contains 7S RNA essential for protein translocation across the ER. *Nature* **299**, 691-698.

Wang AM, Creasey AA, Ladner MB, Lin LS, Strickler J, Van Arsdell JN, Yamamoto R, Mark DF. (1985) Molecular cloning of the cDNA for human TNF. *Science* **228**, 149-154.

Webb CF, Tucker PW, Dowton SB. (1989) Expression and analyses of serum amyloid A in the Syrian Hamster. *Biochemistry* **28**, 4785-4790.

Weinstein JA, Taylor JM. (1987) interleukin-1 and the acute-phase response: induction of mouse liver serum amyloid A mRNA by murine recombinant IL-1. *J. Trauma* **27**, 1227-1232.

Weigel D, Jackle H. (1990) The *fork head* domain: a novel DNA binding motif of eukaryotic transcription factors? *Cell* **63**, 455-456.

Weissenbach J, Chernajovsky Y, Zeevi M, Shulman L, Sorecq H, Nir U, Wallach D, Perricaudet M, Tiollais P, Revel M. (1980) Two IFN mRNAs in human fibroblasts: *in vitro* translation and *E. coli* cloning studies. *Proc. Natl. Acad. Sci. USA* **77**, 7152-7156.

Whitehead AS, Rits M. (1989) Characterization of the gene encoding mouse serum amyloid P component. *Biochem. J.* **263**, 25-31.

Whitehead AS, Zahedi K, Rits M, Mortensen RF, Lelias JM. (1990) Mouse C-reactive protein. *Biochem. J.* **266**, 283-290.

Wiebauer K, Gumucio DL, Jones JM, Caldwell RM, Hartle HT and Meisler MH. (1985) A 78kb region of mouse chromosome 3 contains salivary and pancreatic amylase genes and a pseudogene. *Proc. Natl. Acad. Sci. USA* **82**, 5446-5449.

Wijelath ES, Kardasz AM, Drummond R, Watson J. (1988) IL1 induced inositol phospholipid breakdown in murine macrophages: possible mechanisms of receptor activation. *Biochem. Biophys. Res. Comm.* **152**, 392-397.

Wilson DR, Juan TS-C, Wilde MD, Fey GH, Darlington GH. (1990) A 58bp region of the human C3 gene confers synergistic inducibility by IL1 and IL6. *Mol. Cell. Biol.* **10**, 6181-6191.

Won K-A, Baumann H. (1990) The cytokine response element of the rat α_1AGP gene is a complex of several interacting regulatory sequences. *Mol. Cell. Biol.* **10**, 3965-3978.

Wong GG, Clark SC. (1988) Multiple actions of IL6 within a cytokine network. *Immunol. Today* **9**, 137-139.

Woo P, Betts J, Edbrooke MR. (1991) The human serum amyloid A genes and their regulation by inflammatory cytokines. *In* "Amyloid and Amyliodosis 1990" (Natvig JB, *et al*, eds.) Kluwer Academic, Dordrecht. pp.13-19.

Woo P, Korenberg JR, Whitehead AS. (1985) Characterization of genomic and complementary DNA sequence of human C-reactive protein, and comparison with the complementary DNA sequence of serum amyloid P component. *J. Biol. Chem.* **260**, 13384-13388.

Woo P, Sipe JD, Dinarello CA, Colten HR. (1987) Structure of a human serum amyloid A gene and modulation of its expression in transfected L cells. *J. Biol. Chem.* **262**, 15790-15795.

Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. (1990) CD14, a receptor for complexes of LPS and LPS binding protein. *Science* **249**, 1431-1433.

Wu C-I, Li W-H. (1985) Evidence for higher rates of nucleotide substitution in rodents than in man. *Proc. Natl. Acad. Sci. USA* **82**, 1741-1745.

Yamamoto K-I, Goto N, Kosaka J, Mitomo K, Shiroo M, Migita S, Nakayama S, Nastuume-Sakai S. (1988) Diverse gene structure for serum amyloid A protein in wild mice. *In* "Amyloid and Amyloidosis" (Isobe T, *et al.* eds) Plenum Press, New York. pp.193-297.

Yamamoto K-I, Goto N, Kosaka J, Shiroo M, Yeul YD, Migita S. (1987) Structural diversity of murine serum amyloid A genes: evolutionary implications. *J. Immunol.* **139**, 1683-1688.

Yamamoto K-I, Shiroo M, Migita S. (1986) Diverse gene expression for isotypes of murine SAA protein during APR. *Science* **232**, 227-229.

Yamasaki K, Taga T, Hirata Y, Yawata H, Kawanishi Y, Seed B, Taniguchi T, Hirano T, Kishimoto T. (1988) Cloning and expression of the human IL6 receptor. *Science* **241**, 825-828.

Yanisch-Perron C, Vieira J, Messing J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103-119.

Yang-Yen H-F, Chambard J-C, Sun Y-L, Smeal T, Schmidt TJ, Drouin J, Karin M. (1990) Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. *Cell* **62**, 1205-1215.

Yonehara S, Ishii A, Yonehara M. (1989) A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of TNF. *J. Exp. Med.* **169**, 1747-1756.

Yu S, Sher B, Kudryk B, Redman CM. (1984) Fibrinogen precursors. Order of assembly of fibrinogen chains. *J. Biol. Chem.* **259**, 10574-10581.

Zabel U, Baeuerle P. (1990) Purified human IκB can rapidly dissociate the complex of the NFκB transcription factor with its cognate DNA. *Cell* **61**, 255-265.

Zabel U, Schreck R, Baeuerle PA. (1991) DNA binding of purified transcription factor NFκB. *J. Biol. Chem.* **266**, 252-260.

Zahedi K, Whitehead AS. (1989) Acute phase induction of mouse serum amyloid P component. *J. Immunol.* **143**, 2880-2886.

Zhang Y, Lin J-X, Yip YK, Vilcek J. (1988) Enhancement of cAMP levels and of protein kinase C activity by TNF and IL1 in human fibroblasts: Role in induction of IL6. *Proc. Natl. Acad. Sci. USA* **85**, 6802-6805.

Zimlichman S, Danon A, Nathan I, Mozes G, Shainkin-Kestenbaum R. (1990) Serum amyloid A, an acute phase protein, inhibits platelet activation. *J. Lab. Clin. Med.* **116**, 180-186.

Zimmer EA, Martin SL, Beverley SM, Kan YW, Wilson AC. (1980 Rapid duplication and loss of the genes coding for the α chains of haemoglobin. *Proc. Natl. Acad. Sci. USA* **77**, 2158-2162.

Zuraw BL, Lotz M. (1990) Regulation of the hepatic synthesis of C1 inhibitor by the hepatocyte stimulating factors interleukin 6 and interferon γ. *J. Biol. Chem.* **265**, 12664-12670.