Tissue Specific Expression of Serum Amyloid A

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Abstract

The human serum amyloid A family of proteins is comprised of two acute phase proteins (SAA1 and SAA2) and one constitutively expressed protein (SAA4). The SAAs are predominantly produced by the liver although, some extra hepatic expresion has been reported. Liver specific of other proteins, such α-1 antitrypsin, is due to the presence of multiple binding sites for transcription factors which are predominantly but not exclusively expressed in the liver known as liver enriched transcription factor. C/EBP, which is known to transcriptionally regulate SAA expression, is one such Factors. Luciferase reporter constructs containing varying lengths of the 5' flanking regions of SAA2 were transfected into hepatic and non-hepatic cell lines. The results indicated that in addition to the C/EBP site a tissue specific element was also present between -2213bp and -2355bp. This fragment was subsequently shown to bind both a ubiquitously expressed nuclear factor and a liver specific factor. Sequence analysis identified consensuus binding sequences for a number of transcription factors including the factor YY1 which is involved in the transcriptional regulation of the rat SAA1 gene. Mutation of the YY1 consensus sequence caused a shift in the protein binding pattern observed in electrophoretic mobility shift assays. Preliminary analysis of the 5 ' flanking region of SAA4 has identified a C/EBP site at position -57bp. These results indicate that as with other liver specific proteins tissues specific expression of SAA is due to multiple elements incuding C/EBP an, in SAA2, an as of yet unidentified element of which the ubiquitously expressed transcription factor YY1 may be a component.

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For Mam and Dad

Publications

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List of Abbreviations

A.A.	Amyloid A
ApoAI	Apolipoprotein AI
APP	Acute Phase Protein
APR	Acute Phase Response
CAM	Cellular Adhesion Molecule
CAT	Chloramphenicol Acetyl Transferase
C/EBP	CCAAT Enhancer Binding Protein
CRP	C reactive Protein
DMEM	Dulbeccos Modified Essential Media
DMS	Dimethylsulphate
ECM	Extra Cellular Matrix
EMSA	Electrophoretic Mobility Shift Assay
HDL	High Density Lipoprotein
HNF	Hepatic Nuclear Factor
IL-1	Interleukin 1
IL-6	Interleukin-6
JCA	Jeuvenile Chronic Arthritis
LCAT	Lecithin Cholesterol Transferase
LPS	Lipopolysaccharide
Luc	Luciferase
NFκB	Nuclear Factor κ Binding
PCR	Polymerase Chain Reaction
PLA2	Phopholipase A2
PMA	Phorbo-12-Myristate-13-Acetate
SAA	Serum Amyloid A
SAP	Serum Amyloid P
T.E.	Tris-EDTA
TMED	N,N,N'N'- Tetramethylethylenediamine
TNF	Tumour Necrosis Factor
YY1	Ying-Yang 1

1. Introduction

1.1 Inflammation

Inflammation is the localised and systemic reaction initiated in response to tissue damage. This damage can be incurred physically by mechanical or chemical agitation and burns, or by an infectious agent. The characteristic signs of inflammation rubor (redness), tumour (swelling), dolor (pain) and calor (heat) were first reported in the first century A.D. *Functio laesa* or loss of function was added to this list in the 19th century by Virchow. These manifestations are attributable to the underlying processes initiated to limit and repair the tissue damage. The localised inflammatory response has three main components, increased blood flow, increased vascular permeability and chemotactic migration. The increased blood flow to the damaged area caused by vasodilation allows an influx of leucocytes and serum proteins, increased vascular permeability enables these cells and proteins to pass through the endothelium followed by chemotactic migration of leucocytes to the damaged tissue. These reactions are initiated and mediated by components of the plasma enzyme systems (clotting, kinin, complement and plasmin), phospholipid metabolites and cytokines.

1.1.1. Mediators of Vascular Changes

1.1.1.1. Vasoactive Amines and the Kinin System

The initial or immediate vascular changes which occur during an inflammatory response are primarily mediated by vasoactive amines and products of the kinin system . Histamine causes vasodilation and vascular permeability. It is produced by mast cells and basophils and is released from cells along with other vasoactive substances, such as 5-hydroxytryptamine (5-HT), by degranulation or as a result of cell death or injury. The complement components C5a and C3a are also vasodilators and increase vascular permeability due to the ability of these components to induce mast cell degranulation as these effects can be blocked by

antihistamine. The kinin system has two principle vasoactive mediators, bradykinin and lysyl-bradykinin which are also vasodilatory and increase vascular permeability. Both of these mediators are cleavage products of the same enzyme but are produced in response to different stimuli. Bradykinin and lysyl-bradykinin are produced from high molecular weight kininogen and low molecular weight kininogen, respectively, by the enzyme kallikrein. This enzyme, in precursor form, is present in both the plasma and in tissues. In the plasma the precursor prekallikrein becomes plasma kallikrein by the actions of activated Hageman factor of the clotting system. The plasma enzyme then converts high molecular kininogen to bradykinin. In the tissues the stimulus is tissue damage which results in the precursor prokallikrein being converted to tissue kallikrein in response to plasmin, intracellular enzymes or plasma kallikrein. Tissue kallikrein acts primarily on low molecular weight kininogen to produce lysyl-bradykinin.

1.1.1.2. Arachadonic Acid Metabolites

Arachadonic acid metabolites, while eliciting many of the same responses of the vasoactive amines and kinin system components, are responsible primarily for regulating later vascular changes, i.e. those occurring from around six hours after the initial stimulus. Arachidonic acid is released from membrane phospholipids by the action of phospholipase A. It is then converted to the prostaglandins, leukotrienes and related products via the cycloxygenase and lipoxygenase pathways. Prostaglandin E is the most common of these metabolites, it can increase vascular permeability and can potentiate the vasodilator effects of histamine and bradykinin.

1.1.2. Leucocyte Migration

1.1.2.1 Adhesion Molecules

The migration of cells from the vessels through the endothelium and to the site of damage is controlled by adhesion molecules and chemotactic factors. Adhesion molecules are expressed on the surface of leucocytes and on the surface of endothelial cells. There are four types or groups of adhesion molecules: cellular adhesion molecules (CAMs); integrin; selectins; and the carbohydrate ligands (Dustin and Springer, 1991., Shimizu *et al*, 1991). The expression of these adhesion molecules differs according to cell population and state of differentiation and activation. The CAMS interact with members of the intergrin family of adhesion molecules. They are members of the immunoglobulin super family and include ICAM-1, ICAM-2 and VCAM. They can all be expressed on endothelium with expression of some CAMS also found on some leucocytes. The integrins include the very late antigens (VLA-1 and VLA-2) and can be found on many cell types including leucocytes. In addition to the CAMS other ligands for the intergrins include extracellular matrix components (VLA-2, VLA-5, VLA-6) lipopolysaccharide (CR3) and immune complexes (CR3, CR4). There are three members of the selectin group. P-selectin, L-selectin and E-selectin. The selectins are found on endothelium and leucocytes. The principle ligands for the selectins are the carbohydrate ligands which tend to be associated with various proteins and of these carbohydrate ligands the oligosaccharides related to sialyl Lewis X appear to be the most common.

1.1.2.2. Chemotactic Factors

Once leucocytes have passed through the vessel endothelium into the extracellular space the presence of chemotactic factors can aid in migration of the cells to the site of injury. Chemotactic molecules include C5a, leukotriene B4 and formyl-methionyl peptides (fMLP). The fMLPs are specific to prokaryotes as, unlike in eukaryotes, translation is initiated with

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a formyl-methionyl residue and hence these peptides would be present as the result of an infectious agent.

1.1.3. Cytokines

As has been previously mentioned the inflammatory response can occur in response to both physical damage and infectious agents. Although the response to these different stimuli is fundamentally the same, there can be differences in activation of the different pathways, order of events and the initiation of the response. The mediators most associated with inflammation are the cytokines Interleukin-1(IL-1) and Tumour Necrosis Factorα (TNF-α). Circulating macrophages that come into contact with an invading pathogen produce and release these cytokines which have numerous effects on the pathways and mechanisms previously described they can cause changes in the presence of adhesion molecules, they are known to upregulate ICAM-1, VCAM-1 and E-selectin. These cytokines stimulate cells to produce a second phase of cytokines of which IL-6 is one along with more IL-1 and TNF. Properties and characteristics of the major cytokines are shown in table 1.1

1.2. The Acute Phase Response

1.2.1 The Acute Phase Response and the Acute Phase Proteins

One of the most notable systemic responses that occurs during the inflammatory response is the change in the circulating levels of a number of plasma proteins These changes are known collectively as the acute phase response and the proteins involved are known as the acute phase proteins . The acute phase proteins fall into two categories: those for which there is found to be a decrease in circulating levels during an acute phase response; and those for which there is an increase. These changes in circulating levels are due to alterations

Table 1.1 Properties and characteristics of the major cytokines. (Reviewed in Male *et al*, 1996. and Nicola *et al*, 1994. Kohno *et al*, 1998., Puren *et al*, 1998). Where no information was available boxes were left blank.

	Major producing cells	Production stimuli	Sites of action	Major biological actions
IL-1	monocytes, fibro-blasts, endothelial cells, macrophages, lymphocytes, keratinocytes, den-dritic cells, astrocytes.	microbial products, TNF, GM-CSF, IL-2, UV light, antigen presentation	thymocytes, neutrophiles, T and B cells, tissue cells.	immunoregulation, acute phase response, fever .
TNFα	macrophages, T cells, B cells, endothelial cells, Kuppfer cells	LPS, viruses, IL-1, IL-2, antigen pre-sentation.	T cells, B cells, macrophages, so-matic tissue cells	acute phase protein induction, bone reabsorption, fever, prostaglanding synthesis
TNFβ	T cells, B cells	mitogenic or anti-genic activation	as TNFα	as TNFα
IL-2	T cells, NK cells	antigen presenta-tion, IL-1, Il-2	T cells, B cells, monocytes	proliferation of T, B, LAK cells, increased cytotoxicity of NK cells
IL-3	T cells	antigen presenta-tion	stem cells, pro-genitors	growth stimuli of pluripotential stem cells
IL-4	T cells	antigen presenta-tion	B cells, T cells	class switching to IgE, IgG production, thymocyte prolifer-ation
IL-5	T cells	antigen presenta-tion	B cells, eosinophiles	differentiation of B cells, en-hances IgA and IgM produc-tion, maturation and growth of eosinophiles
IL-6	monocytes, macrophages, T cells, fibroblasts,glioblasto ma cells	IL-1, TNF, PDGF	T cells, B cells, thymocytes, hepatocytes	proliferation, differentiation induction of immunoglobulin production, acute phase re-sponse
IL-7	stromal adherent cell from bone marrow	unknown	T cells, B cells	growth of pre and pro B cells, T cell activation
IL-8	monocytes, macrophages, fibro-blasts, epithelial cells, kupffer cells	IL-1, LPS, TNFα, IL-3, GM-CSF	granulocytes, T cells	neutrophile chemotaxis and activation, T-call and macro-phage chemotaxis

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IL-9	T cells	antigen presenta-tion	mast cells, B cells, T cells	proliferation of mast cells in synergy with IL-3, enhances IL-4 induced IgE and IgG pro-duction, enhancement of suvival of T helper cells.
IL-10	Th2 cells, mast cells, monocytes, macrophages	antigen presenta-tion	macrophages, NK cells, thymocytes, T cells	macrophage dependent inhibi-tion of IL-2 productions, inhib-itor of immune reponse
IL-11	bone marrow, stromal fibroblasts, trophoblasts		megakaryocytes, hepatocytes	megakaryocyte maturation, acute phase protein synthesis, expansion of megakaryocyte colonies in synergy with IL-3
IL-12	monocytes, macrophages, B cells.		T cells, NK cells	Induction of IFNγ produciton by T and NK cells, differentia-tion of Th1 cells from progeni-tors
IL-13	T cells	LPS,calcium ionophore, phorbol ester	B cells, mono-cytes, macrophages,	B cell proliferation and class switching to IgE, inhibition of proinflammatory cytokine pro-duction
IL-14	T cells, some malig-nant B cells		B cells	proliferation of B cells, inhibi-tion of antibody production, induction of FAS on B cells
IL-15	fibroblasts, activated monocytes		T cells, B cells	T and B cell proliferation, skel-etal muscle hypertrophy
IL-16	T cells (CD8+), endo-thelial cells		T cells (CD4+), monocytes, eosinophils	chemotaxis and activation of T cells(CD4+), monocytes and eosinophils,
IL-17	T cells (CD4+)		T cells, fibroblasts	T cells proliferation, stimulates IL-6 secretion and ICAM 1 in fibroblasts, activates NF-κB
IL-18	T cells		T cells, fibroblasts, macrophages	induction of IFNγ and TNF

in the level of synthesis by the liver which is the predominant site of synthesis of the acute

phase proteins (APPs)

1.2.1.1. Functions of the Acute Phase Proteins

The functions which have so far been attributed to the acute phase proteins are related to

inhibiting, limiting and repairing tissue damage and can be grouped into three categories:

host defence mechanisms; inhibition of serine proteinases; and transport proteins with antioxidant

activity .

1.2.1.2. The Negative Acute Phase Proteins

During an acute phase response circulating levels of certain proteins synthesised in the liver

are decreased. These proteins are known as the negative acute phase proteins. They include

albumin, transthyretin, α-fetoprotein and transferrin (Werner and Cohen, 1969., Dicksen

et al, 1982., Moody et al, 1982). One possible reason for these observed decreases could

be that these proteins are not needed or are not as important under the circumstances and

decreasing their synthesis would give the liver the extra capacity it needs to synthesise the

positive acute phase proteins.

1.2.1.3. The Positive Acute Phase Proteins

The positive acute phase proteins increase in circulating levels during an acute phase response.

The acute phase proteins can be divided into groups. One classification system defines three

classes of positive acute phase proteins according to the degree of increase in circulating

levels which occurs during an acute phase response (Kushner 1982). Class I acute phase

protein are those for which there is an approximately 50% increase, class II proteins are

those for which there is approximately a 2-10 fold increase in circulating levels and class

III proteins are those for which there is a greater than 10 fold increase in protein levels. A more recent classification system, and one which is now more commonly used, is to classify the acute phase proteins by there responsiveness to cytokines (Bauman and Gouldie, 1994). The increase in acute phase protein levels which occurs during a response is predominantly due to increased expression by the liver. This increased expression is due to the presence of cytokine responsive regulatory mechanisms within the genes encoding the proteins. The acute phase protein genes can be divided into two groups depending on their cytokine responsiveness. Type I genes were initially considered to be responsive to IL-1 and TNF but it is now known that these genes are also responsive to the IL-6 family of cytokines which act cooperatively with the IL-1 and TNF cytokines to produce a maximal response. Type II acute phase genes are responsive only to the IL-6 family of cytokines, IL-1 and TNF have no activational or repressive effect on the genes. The Type I acute phase genes include those encoding the APP's for which the greatest increases in circulating levels are found, SAA and C-reactive protein, and the moderately increased complement component C3. It is the combined effects of both IL-1 (or TNF) and IL-6 family of cytokines which allows such dramatic increases in protein levels. The cytokines bind to their respective receptors on the cell surface which initiates a signalling mechanism the result of which is binding of transcription factors to recognition sequences within the gene.

1.3. Cytokines and Acute Phase Gene Induction

1.3.1. Interleukin 1 and Tumour Necrosis Factor

1.3.1.1. Interleukin 1

IL-1 is produced following injury or infection. The major producers of IL-1 are activated macrophages although it can also be produced by a number of other cell types including T and B cells, fibroblasts, keratinocytes, brain astrocytes and microglial cells. It is pleiotrophic,

exhibiting a number of biological effects (see table 1.1). IL-1 is involved in B cell activation, stimulates T-cell proliferation, induces production of acute phase proteins, can induce fever and cause bone resorption and cachexia. IL-1 has also been implicated in the pathogenesis of a number of inflammatory diseases including rheumatoid arthritis (Wood *et al*. 1983, Duff.1985)

There are two distinct forms of IL-1: IL-1α and IL-1β (Auron *et al*, 1984., March *et al*, 1985), both of which are produced as 31KDa precursor proteins: proIL-1α and proIL-1β. These precursors are cleaved at approximately residue 115 to leave the C terminal 100 amino acids as the mature protein. IL-β, the predominant form in humans, is cleaved from it's precursor to the active form by ICE (IL-1 converting enzyme). As well as the mature proteins IL-1α and IL-1β the IL-1β precursor protein (pro IL-1β) is also an active protein (Rosenwasser et al, 1986., Jobling et al, 1986). ProIL-1α is not biologically active. IL-1α , unlike IL-1β , is predominantly found bound to the cell surface. A third member of the IL-1 family is IL-1 receptor antagonist which binds to IL-1 receptors preventing binding of IL-1α and IL-1β and hence inhibiting biological activity (Arend *et al*, 1985., Arend *et al*, 1991).

1.3.1.2. IL-1 Receptors

The IL-1α and IL-1β isoforms are only 16% homologous yet they bind to the same receptors and exhibit the same biological activities. There are two IL-1 receptors, IL-1R type I and IL-1R type II (Sims *et al*. 1988, McMahon *et al* . 1991). Only the type I receptor is capable of signalling (Stylianou *et al*, 1992., McKean *et al*, 1993., Sims *et al*, 1993.). The type II receptor functions as a decoy. The IL-1 type I receptor is an 80 KDa polypeptide chain. It has three distinct regions, a cytoplasmic region of 210 amino acids , a transmembrane region of 22 amino acids, and an extracellular domain of 317 amino acids. The extracellular

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domain is made up of three immunoglobulin motifs and the type 1 receptor is therefore classed as a member of the immunoglobulin family (Urdal *et al.* 1988). The type II receptor also contains three immunoglobulin motifs and is a member of the immunoglobulin family. It has a short cytoplasmic region compared to the type I receptor of just 29 amino acids. IL-1R type II also has a transmembrane region of 22 amino acids and an extracellular domain of 334 amino acids. The three members of the IL-1 family can all bind both the IL-1R type I and type II, the type II receptor preferentially binds IL-1β (Kilian *et al.* 1986., Dower et al, 1992).

1.3.1.3. Tumour Necrosis Factor

As with IL-1 TNF has two forms α and β. The two forms are closely related and share many biological properties (see table 1.1). TNF-α is produced predominantly by macrophages but also by natural cytotoxic cells, T and B cells, granulocytes, fibroblasts, mast cells, adipocytes and others. TNF-α and IL-1 share several biological properties such as induction of fever , stimulation of collagenase production and stimulation of acute phase protein synthesis (a property also shared with IL-6). TNF-α exists under native conditions as a trimeric molecule (Aggarwal, 1985). Each subunit is a single polypeptide chain of 157 amino acid residues with a molecular weight of approximately 17kDa and has an antiparallel β-sheet sandwich conformation (Beutler, 1992., Eck and Sprang, 1989). The biologically active form of TNF-β is also a trimeric molecule with an approximately 25kDa molecular weight for each subunit. Unlike TNF-α it has no transmembrane region and is therefore not found membrane bound but is secreted. TNF-β is produced by lymphocytes due to viral or antigenic stimuli and is tightly regulated. Functionally it has cytotoxic properties and it can promote cell proliferation and differentiation.

1.3.1.4. TNF Receptors

There are two forms of the TNF receptor, p80 and p60. The p80 TNF receptor has an extracellular domain of 235 amino acids, a transmembrane domain of 30 amino acids and a cytoplasmic domain of 174 amino acids. It has two possible N glycosylation sites and several possible O glycosylation sites. The p60 form has an extracellular domain of 182 amino acids, a transmembrane domain of 21 amino acids and a cytoplasmic domain of 223 amino acids (Fuchs et al, 1992). In contrast to the p80 receptor it has no O glycosylation sites but it does have three N linked glycosylation sites (Dembic et al ,1990). The extracellular domains of the two receptors each have four cysteine rich domains. The cell surface expression of the TNF receptors is regulated by a number of agents including the cytokine IL-1, IL-6 and TNF itself.

1.3.1.5. IL-1 and TNF Signalling Pathways

Although IL-1 and TNF bind to different receptors they exhibit similar biological properties which can be attributed to the fact that the same signalling pathways are being utilised by different cytokine-receptor complexes. The mechanisms involved in the initial stages in IL-1 and TNF signalling have been the subject of much speculation and controversy, G-proteins (Chedid *et al.* 1989,O'Neill *et al.* 1990), cAMP-dependent kinase/PKA (Chedid and Mizel ,1990., Zhang *et al*, 1988). and protein kinase C (Munoz et al, 1990,. Guy et al, 1992) have all been implicated at some time. Within recent years the sphingomyelin pathway has also been identified as a mechanism utilised for the transduction of the IL-1 signal. Sphingomyelin is present in the outer leaflet of the plasma membrane of most mammalian cells and can be hydrolysed to ceramide by the enzyme sphingomyelinase. Ceramide can transverse the lipid bilayer and act as a second messenger. Kinase activity has been associated with IL-1 signalling as IL-1 is known to increase the phosphorylation of a number of cell proteins.

This is most commonly serine and threonine phosphorylation. A number of different kinases have been implicated in IL-1 signalling. They can be associated with different pathways and can also form kinase cascades (the involvement of particular kinases will be discussed further in the following sections). Although much is known about IL-1 signalling there is still much to discover and clarify. However what is known is that ultimately these signalling pathways lead to the activation of the transcription factors NF-κB (Osborn *et al*, 1989) and AP-1 (McKean *et al*, 1994; Bauman *et al*, 1991) primarily as well as NF-IL-6 (C/EBPβ) (Isshiki *et al*, 1991) which is also an IL-6 inducible transcription factor (see section 1.3.2.9.). It is these transcription factors which are responsible for mediating the biological effects of IL-1.

1.3.1.6. NF-κB

The transcription factor NF-κB was originally identified by Sen and Baltimore (1986) as a factor which bound the κ light chain enhancer in B cells. NF-κB has been established as an inducible transcription factor involved in regulating the expression of a number of genes associated with inflammation and the acute phase response. These include the cytokines IL-2, IL-6, IL-8 and the adhesion molecule ICAM-1 (Ledeburh and Parks, 1995). This involvement in the immune response means that NF-κB could be involved in the pathogenesis of a number of conditions. NF-κB is known as an immediate early response factor as it is activated within minutes of ligand binding to the IL-1 type I receptor. The reason for this rapid activation is that it is independent of *de novo* protein synthesis. NF-κB is present within the cytosol complexed to an inhibitory protein IκB (Baeuerle and Baltimore, 1998). The IκB binds to NF-κB masking the nuclear localisation sequences preventing translocation of NF-κB to the nucleus (Beg *et al*, 1992., Henkel *et al*, 1992). In order for the NF-κB to translocate to the nucleus the IκβΒ is degraded following phosphorylation by Iκ B kinase (IKK) (fig 1.1). Although all of the signalling intermediates which lead to degradation of

IκB have not yet been elucidates it is known that upon binding of IL-1α orIL-1β to the IL-1 type I receptor, a recently described IL-1R accessory protein (IL-1RAcP) associates with the IL-1RI (Greenfeeder *et al*, 1995). The IL-1RAcP increases the affinity of the receptor for IL-1β. The binding proteins TNF Receptor Associated Factor (TRAF) and MyD88 are recruited to the receptor. These proteins associated with kinases which do not phosphorylate IκB kinase directly but appear to be part of a cascade. They are NF-κB-inducing kinase (NIK), IL-1 receptor associated kinase (IRAK) and IRAK-2 (Croston *et al*, 1995). This eventually leads to targeted phosphorylation of the IκB and ubiquitination which allows degradation of the IκB by proteosome. With the IκB degraded and released from the nuclear localisation sequences the NF-κB is translocated to the nucleus. Multiple forms of both NF-κB and IκB have been identified. NF-κB exists as a homo or heterodimer composed of two subunits each of which is a member of the NF-κB/*rel* family of proteins. Identified subunits are c-*rel*, *Rel*A (p65), *Rel*B, p50 and p52 which all have a 300 amino acid homologous region known as the rel homologydomain (Nolan *et al*, 1991; Ryseck *et al*; Neri *et al*, 1991) The p50 and p52 proteins exist initially as precursor proteins known as NF-κB1(p105) and NF-κB2 (p100). These precursor proteins contain regions of ankyrin repeats which are also present in the inhibitory IκB proteins and these regions must be cleaved to leave a functional subunit. The originally identified NF-κB was composed of subunits p50 and p65 and is the most common form of NF-κB(Urban *et al*, 1991). Multiple forms of IκB have also been identified: IκBα, IκBβ, and recently identified IκBγ (Haskill *et al*, 1991., Baeuerle and Henkle, 1994., Siebenlist *et al*, 1994). It has been reported that the degradation of the α and β forms of IκB could be via different pathways as it has been found that although IL-1, TNF, LPS and PMA stimulate the degradation IκBα, IL-1 and LPS but not TNF and PMA stimulate degradation of IκBβ. Once translocated to the nucleus the NF-κB activates transcription of the target genes upon binding to DNA. In addition to containing DNA binding domains some of the rel proteins are able to bind other transcriptional activators. *Rel* A and c-*rel*

can interact with TATA binding protein (TBP) and *Rel A* can interact with TFIIB.
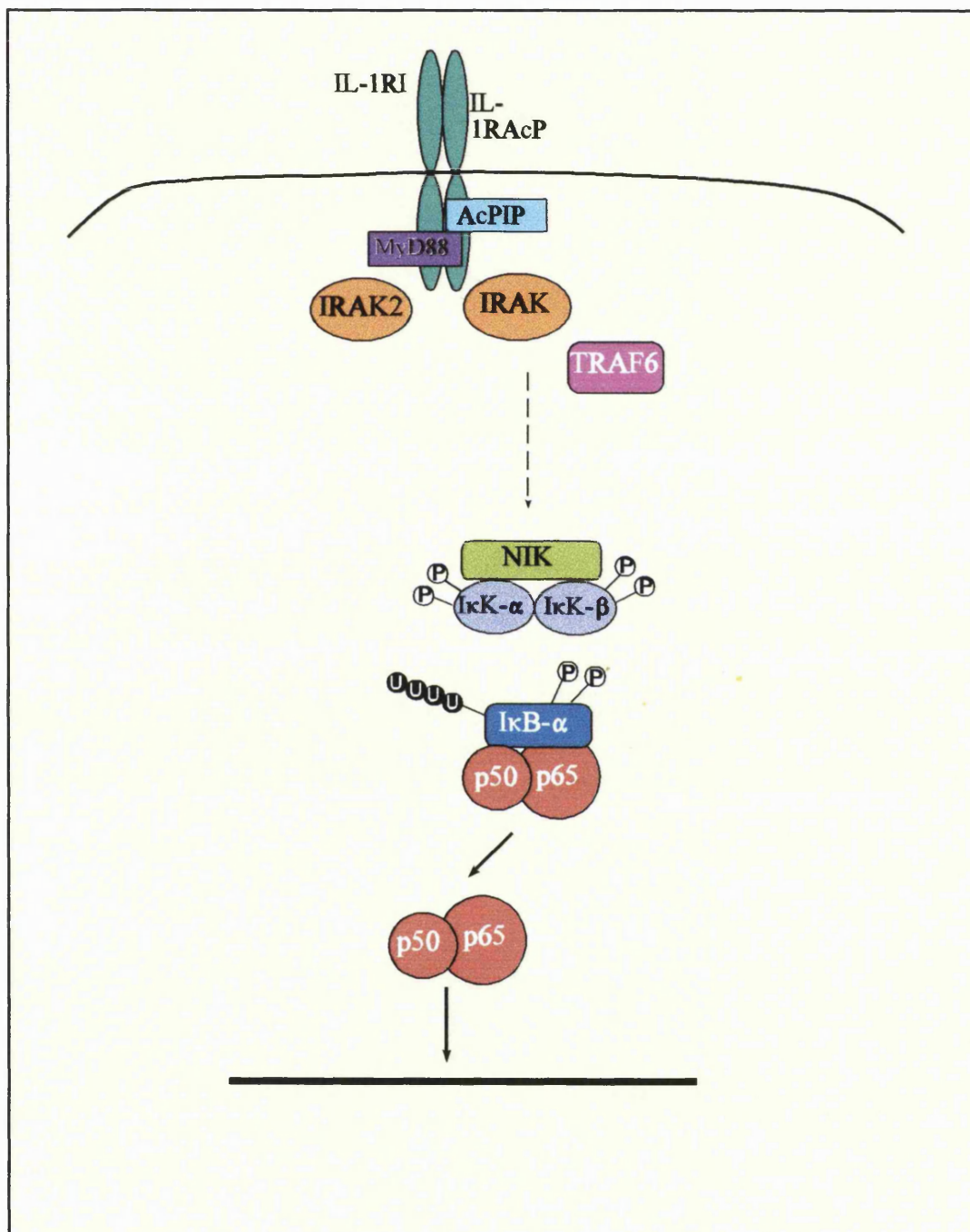
Figure 1.1. Activation of NFκB in response to IL-1 receptor binding ligand. Phosphorylation and Ubiquitination are represented by P and U respectively. For all other abbreviations refer to section 1.3.1.6.

1.3.1.7. AP-1

AP-1 is also an IL-1 inducible complex which is involved in the transcriptional regulation of a number of genes associated with the immune response (Moshage, 1997).AP-1 is a dimeric protein made up of members of the *jun* and *fos* polypeptide families. The *Jun* polypeptides are c-*Jun*, *Jun*B and *Jun*D. The *Fos* polypeptides are c-*Fos*, *Fos*B, *Fra*-1 and *Fra*-2. The AP-1 dimeric complex can be a *Jun* homodimer, a *Jun* heterodimer or a *Jun/Fos* heterodimer. AP-1 is activated in response to IL-1 via a pathway involving ERK and JNK kinases.

1.3.2. IL-6 family of Cytokines

The IL-6 family of cytokines are IL-6, leukaemia inhibitory factor (LIF), IL-11, oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and cardiotrophin-1 (CT-1). These cytokines share little sequence homology but do appear to have similarities in their secondary structures(Bazan, 1991). The receptors for the IL-6 family of cytokines are composed of a cytokine binding chain and a non-cytokine binding signal transducing chain. The signalling receptor called gp130 is common to all IL-6 family receptors. The cytokine binding chain is specific to each cytokine and each shows differential tissue distribution.As a result the IL-6 family of cytokines can can exhibit different biological properties according to the cells upon which each receptor is expressed.

1.3.2.1. Interleukin-6

IL-6 is produced by a variety of cells including T cells, macrophages, fibroblasts and astrocytes (see table 1.1). It is a pleiotrophic cytokine whose effects include induction of immunoglobulin secretion in B cells, cytotoxic T cell differentiation and induction of acute phase protein synthesis. IL-6 has been implicated in the pathogenesis of rheumatoid arthritis (Hirano et

al 1988., Bhardwaj et al, 1989., Castlemans disease (Yoshizaki et al, 1989) and other disorders. IL-6 is a 184 amino acid glycoprotein containing two disulphide bridges. It has a molecular weight of 21-26KDa depending on post translational modifications which are N and O linked glycosylation and phosphorylation (Santhanam *et al*, 1989).

1.3.2.2. Leukaemia Inhibitory Factor (LIF)

LIF is a basic monomeric protein with a molecular weight of 32 to 62 kDa when produced which is reduced to 20 to 25 kDa upon deglycosylation . It can be produced by a wide range of tissues. *In vitro* effects of LIF include the ability to induce the differentiation and suppress the clonogenicity of the mouse monocytic leukaemia cell line M1, suppression of the clonogenicity of the human leukaemic cell lines HL-60 and U937 and suppression of embryonic stem cell differentiation (Gearing et al, 1987., Williams et al, 1988). *In vivo* effects as demonstrated using over expressing and non-expressing mouse models indicate that LIF is involved in maintaining pregnancy as mice lacking LIF are unable to carry to term (Stewart et al, 1992). Over expression of LIF resulted in wasting, elevated platelets levels and aberrant bone deposition (Metcalfe, 1992).

1.3.2.3. Oncostatin M (OSM)

OSM is a monomeric glycoprotein with a molecular weight of 28kDa (Zarling et al, 1986). Produced as a 252 amino acid precursor the mature protein is formed by removal of a 25 amino acid leader sequence and 31 amino acids from the C-terminal end of the protein (Malik et al, 1989). OSM is produced by activated monocytes and T lymphocytes and has a range of biological functions (Bruce et al, 1992). Activities demonstrated *in vitro* include inducing the differentiation of leukaemic cells to macrophage like cells, inhibition of differentiation of embryonic stem cells and stimulation of growth of fibroblast cells or cells from AIDS-related Kaposi's sarcoma.

1.3.2.4. IL-11

IL-11 is a 178 amino acid monomeric protein with a molecular weight of 19 kDa (Paul et al, 1990). Its biological activities include promoting megakaryocyte differentiation , growth of erthyroid colonies and inhibition of adipocyte differentiation (Kawashima et al, 1991., Burstein et al, 1992., Quesniaux et al, 1992). IL-11 can be produced from fibroblast cells derived from bone marrow or lung and has been found to be produced by cells from a thyroid malignancy.

1.3.2.5. Ciliary Neurotrophic Factor (CNTF)

CNTF is predominantly produced by the schwann cells in peripheral nerves but is also produced at lower levels in adult brain, skeletal muscle and in the embryo (Rende et al, 1992., Sentner, 1992., Ip et al, 1993). A role for CNTF in limiting or repairing injury has been put forward as it has been following injury to the nervous system schwann cells close to the site of damage release cytoplasmically stored CNTF into the extracellular space (Friedman et al, 1992., Rende et al, 1992., Sendtner et al, 1992). CNTF has also been found to inhibit proliferation and promote differentiation of neurone precursors and be involved in the survival and maturation of oligodendrocytes (Loius et al, 1993).

1.3.2.6. IL-6 Receptor

The IL-6 receptor consists of two polypeptide chains, a receptor and a signal transducer (fig 1.2). The human IL-6 binding receptor is a glycoprotein which has a 339 amino acid extracellullar domain, a 28 amino acid transmembrane region and an 82 amino acid cytoplasmic region . The IL-6 receptor chain has a molecular weight of 80KDa which is 30 KDa above

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the expected size due to N-glycosylation. The chain contains one immunoglobulin like region but is structurally closer to the haemopoetic receptor family (Yamasaki *et al*, 1988). The second component of the IL-6 receptor complex is gp130. The human gp130 has a 597 amino acid extracellular domain, a 22 amino acid transmembrane domain and a cytoplasmic region of 277 amino acids (Hibi *et al*, 1990).The gp130 protein has a molecular weight of 130kDa which is approximately 20kDa higher than the predicted size due to glycosylation. As with the IL-6 binding receptor this signalling receptor is also structurally similar to members of the haemopoetic receptor family. However unlike the IL-6 binding receptor gp130 does not bind ligand. IL-6 binds to the IL-6 receptor with low affinity. This binding induces homodimerisation of gp 130 (Taga *et al*, 1989).

1.3.2.7.Other IL-6-like Cytokine Receptors

The signal transducing chain gp130 is common to all the IL-6 family cytokine receptors and forms either a homodimer or a heterodimer with the structurally related receptor chain LIF-binding protein (LIFBP). The LIF and OSM receptors are both composed of a gp130/LIFBP heterodimer (Ip et al, 1992., Taga et al, 1992). CNTF binds to its receptor CNTFR which is homologous to the IL-6R which results in formation of a gp130/LIFBP heterodimer (Davis et al, 1993). It is thought that the differences in biological effects of these cytokines and the cell type specificity to which some of these effects can be attributed could be due to restricted expression of different binding receptors to certain cells types.
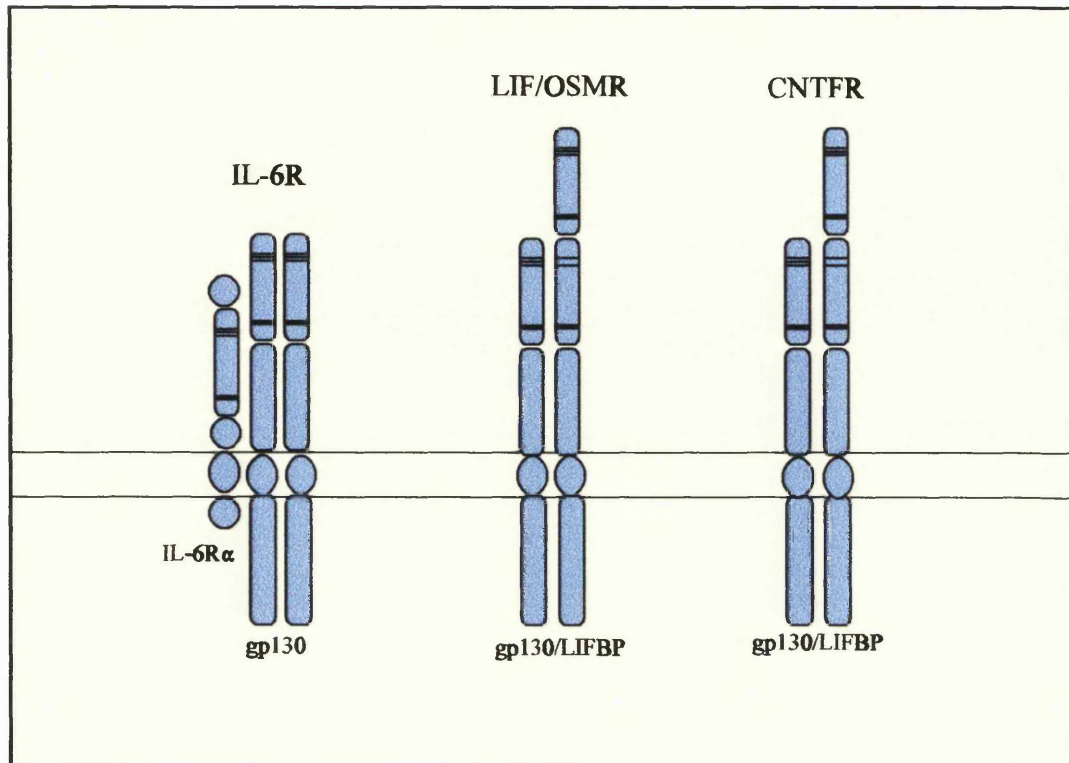
Figure 1.2. The IL-6 family of cytokine receptors. IL-6R-interleukin 6 receptor, LIF-leukaemia inhibitory factor, OSMR-oncostatin M receptor, CNTFR-cilliary neurotrophic factor receptor, LIFBP-leukaemia inhibitory factor binding protein.

1.3.2.8. IL-1 Signalling Pathway

Activation of the IL-6 receptor complex results in activation of two pathways. Upon IL-6 binding, JAK tyrosine kinases phosphorylate tyrosine residues on signal transducers and activators of transcription (STAT) proteins, this includes STAT3 formerly known as APRF (Akira et al, 1994., Stahl et al, 1994). This induces homodimerisation of the STAT proteins which then translocate to the nucleus where they recognise and bind motifs known as IL-6 response elements which are present in the genes of type II acute phase protein genes. In addition the IL-6 receptor complex can also activate the MAP kinase pathway resulting in upregulation of members of the C/EBP family of transcription factors (Nakajima et al, 1993), which recognise and sites present in type I acute phase protein genes and also type II acute phase genes.

1.3.2.9. CCAAT Enhancer Binding Protein (C/EBP)

The C/EBPs are a family of transcription factors. C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ and C/EBPε have been identified so far. Each factor is encoded by a different gene. The C/EBP transcription factors bind to DNA as either homodimers or heterodimers. They all contain a leucine zipper motif through which they dimerise at the C-terminal of the protein, adjacent to the DNA binding region (Landshulz *et al*, 1988).The original or first described member of the C/EBP is C/EBPα (Johnson *et al*, 1987). C/EBPβ was originally termed NF-IL-6 and was identified as IL-1 inducible factor (Issiki *et al*, 1990) It was found to bind and mediate transcription of the IL-6 gene hence the name nuclear factor IL-6. C/EBPδ was previously known as NF-IL-6β. C/EBPβ and C/EBPδ are present at low levels under normal condition but are induced in response to Il-1,TNF and IL-6. Not all members of the family are inducible, C/EBPα is constitutively expressed (Akira,1992).Upon ligand binding gp130 asociates with the IL-6 receptor (Taga *et al*, 1989).This is followed by tyrosine phosphorylation of gp130 which is known to associate with Janus kinases (JAK) (Stahl *et al*, 1994). The result is initiation of signalling pathway which involves the Mitogen Activated Protein (MAP) kinase cascade. C/EBP will be discussed further in section 1.6.1.

1.4. The Major Human Acute Phase Proteins

The major acute phase proteins are Serum Amyloid A (SAA) and either C-reactive protein or serum amyloid P (SAP) depending upon the species. The major acute phase proteins are those for which the greatest increases in circulating levels are found and which can be as much as a 1000 fold increase over 'normal' levels. Although a number of effects have been observed for the major acute phase proteins their functions have yet to be clearly defined and they remain the least well understood with regards to function of all the acute phase proteins.

1.4.1. C-reactive Protein and Serum Amyloid P (SAP)

Although C-reactive protein and SAP have been found to be expressed simultaneously in the same animal species only one of the two proteins is an acute phase protein in any one species. In humans, C-reactive protein is an acute phase protein whereas in mice it is SAP which is the acute phase protein (Whitehead *et al*, 1983., Mantzouranis *et al*, 1985., Floyd-Smith *et al*, 1986). C-reactive protein and SAP are both located on the same region of chromosome 1. They are also both pentraxins, by definition composed of five identical globular subunits arranged in a disc formation. C-reactive protein has a single disc structure while SAP has a double disc structure. It is thought that these two proteins could be derived from a common ancestor which duplicated at around 200 million years ago or earlier (Rubio *et al*, 1993). Interestingly it has been found that in mice transgenic for the human C-reactive protein gene, expression of the transgene could be induced indicating that the transgene was 'acute phase' in nature in the mouse and that inability of the mouse gene to respond during an acute phase response may lie in differences in the sequence of the gene and not in other components of the inflammatory response.

C-reactive protein was originally identified as a protein which bound to the C-polysaccharide of pneumococcus and precipitated in the sera of patients with pneumococcal infections (Tillet and Francis, 1930.). It exhibits a high degree of evolutionary conservation, this extends to the horseshoe crab for which there has been shown to be a great deal of sequence similarity with the human gene (Robey and Liu, 1981). Functional properties which have so far been identified for C-reactive protein include opsonisation, complement activation (Volankis and Kaplan, 1970; Clauset *et al*, 1977) and modulation of chemotactic activity (Buchta *et al*,.1987). It can also bind chromatin, histones and small nuclear ribonuclear particles possibly in a role to remove them after they have been released from damaged cells (Robey *et al*, 1985., Ducloset *et al*, 1988., Shephard *et al*, 1986.)

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Serum Amyloid P is an acute phase protein in mice but not in humans. It is found as a circulating protein, and is present in all types of amyloid of which it comprises up to 15 % of the mass of amyloid fibrils in vivo. (Skinner and Cohen, 1988) (see section 1.5.3.1.). AP, which is identical to SAP, is an extrafibrillar glycoprotein associated with the basement membrane. It also shares a number of biological properties with C-reactive protein including binding of histones and other nuclear material and activating of complement. In contrast to CRP and SAP, the other major acute phase protein serum amyloid A, is an acute phase protein in all species in which it is present. However as will be discussed in section 1.5.1.1. multiple isoforms of SAA exist and not all are acute phase proteins.

1.5. Serum Amyloid A

1.5.1. Amyloid A protein

Serum amyloid A is an apolipoprotein which circulates predominantly as part of high density lipoprotein fraction 3 (HDL_3) particles (Benditt and Ericksen, 1977., Meek *et al*, 1992., Whitehead *et al*, 1992). Some SAA has also been found associated with other HDL fractions and with low density lipoprotein (LDL) and very low density lipoprotein (VLDL) (Marhaug *et al*, 1982). SAA is not one but a family of 14KDa proteins which contains both constitutively expressed proteins and acute phase proteins. The acute phase SAA proteins are present in the circulation at low levels (1 to 5 μg/ml) during normal circumstances however during an acute phase response the circulating levels can increase up to 1000 fold. As a result the percentage of the total protein content of HDL which is SAA increases from 1% to 50%.

HDL_3 is composed of a polar lipid surface layer which contains phospholipids, cholesterol and apolipoproteins and has a non-polar lipid core which contains triglycerides and cholesterol esters. To be integrated into the HDL_3 particle SAA would have to have an amphipathic

region. It has been found that the strongest lipid binding region within the SAA molecule is contained within the first 11 N terminal residues. This is supported by the fact that protein AA, for which SAA is the precursor, is also associated with HDL and yet it lacks the 28 C-terminal residues. Analysis of the amino acid residues within the SAA molecule reveals that residues 75-104 in human acute phase SAA contain no lipid binding sequences. Mutation of SAA by deletion of amino acids 1-11 prevents association with HDL confirming that this is the lipid binding region (Patel *et al*, 1996).

1.5.1.1. Protein Sequence and Secondary Structure of SAA

In humans two acute phase protein isoforms, SAA1 and SAA2 have been identified along with one constitutively expressed isoform, SAA4. The SAA1 and SAA2 isoforms are 95% homologous at the amino acid level but show only 50% homology with SAA4. SAA is conserved throughout a number of species (fig 1.3). Infact the human SAA1 and SAA2 isoforms are more homologous to the mouse acute phase isoforms than to the human SAA4 variant. The human acute phase SAA isoforms are composed of 104 amino acid residues with a 18 amino acid leader sequence. The human SAA1 and SAA2 genes are polymorphic producing protein variants (Parmelee *et al*, 1982 and Betts *et al*, 1991). The proteins SAA1α and SAA1β differ in that SAA1α has a valine at position 52 and an alanine at position 57 where as SAA1β has an alanine at position 52 and a valine at position 57, SAA1γ has alanines at residues 52 and 57. The SAA2α and SAA2β isoforms differ in that SAA2α has a His at position 71 whereas SAA2β has a Arg. The SAA isoforms can also undergo posttranslational modification to produce isoforms in which the amino terminal arginine has been removed. The constitutive form of SAA, SAA4, is composed of 130 amino acid residues which includes an 18 amino acid leader peptide. SAA4 is considerably different at the amino acid level. The main difference is the insertion of an octapeptide at residues 70 to 77(Watson *et al*, 1992). This insertion is also present in the acute phase isoforms

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                  1                              50                                  100
human4      MRLFTGIVFCSLVMGVTSESWRSFFKEALQGVGDMGRAYWDIMISNHQNS NRYLYARGNYDAAQRGPGGVWAAKLISRSRVYLQGLIDYYL.FGNSSTVL EDSKSNEKAEEWGRSGKDPDRFRPDGLPKKY
mouse4      MRLATVIVLCSLFLGVSGDGWYSFFREAVQGTWDLWRAYRDNLEANYQNA DQYFYARGNYEAQQRGSGGIWAAKIISTSRKYFQGLLNRIY.FGIRGQGQ EDSRADQAANEWGRSGKDPNHFRPAGLPDKY
------------------------------------------------------------------------------------------------------------------------------------------
cow         ~~~~~~~~~~~~~~~~~~~~Q.WMSFFGEAYEGAKDMWRAYSDMREANYKGA DKYFHARGNYDAAQRGPGGAWAAKVISDARENIQRFTDPLFKGTTSNHGL ETLQATQKAEEWGRSGKNPNHFRPEGLPEKF
sheep       ~~~~~~~~~~~~~~~~~~~~Q.WLSFLGEAYEGAKDMWRAYSDMREANFKGA DKYFHARGNYDAAQRGPGGVWAAEVISNGREALQGITDPLFKGMTRDQVR EDTKADQFANEWGRSGKDPNHFRPPGLPDKY
horse       ~~~~~~~~~~~~~~~~~~~~~LLSFLGEAARGTWDMIRAYNDMREANYIGA DKYFHARGNYDAAKRGPGGAWAAKVISDARENFQRFTD.RFSFGGSGRGA EDSRADQAANEWGRSGKDPNHFRPHGLPDKY
horse1      ~~~~~~~~~~~~~~~~~~~~~LLSFLGEAARGTWDMLRAYNDMREANYIGA DKYFHARGNYDAAKRGPGGAWAAKVISDARENFQRFTD.RFSFGGSGRABZ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
mink1       MKLFTGLIFCSLVLGVSSQ.WYSFIGEAAQGAWDMYRAYSDMIEAKYKNS DKYFHARGNYDAAQRGPGGAWAAKVISDARERSQRITD.LIKYGDSGHGV EDSKADQAANEWGRSGKDPNHFRPPGLPDKY
mink2       MKLFTGLIFCSLVLGVSSQ.WYSFIGEAVQGAWDMYRAYSDMREANYKNS DKYFHARGNYDAAQRGPGGAWAAKVISDARERSQRVTD.LFKYGDSGHGV EDSKADQAANEWGRSGKDPNHFRPSGLPDKY
dog         MKLFPGLLFCSLVLGVSGQ.WYSFVSEAAQGAWDMWRAYSDMREANYKNS DKYFHARGNYDAAQRGPGGAWAAKVISDARENSQRITD.LLRFGDSGHGA EDSKADQAANEWGRSGKDPNHFRPAGLPDKY
mink        ~~~~~~~~~~~~~~~~~~~~Q.WYSFFGEAIQGAWDMYRAYSDMREANYKNS DKYFHARGNYDAAQRGPGGAWAAKVISDARERQQRITD.LIK.GDS........KADQAANEWGRSGKDPNHFRPPGLPDKY
cheetah     ~~~~~~~~~~~~~~~~~~~~E.WYSFLGEAAQGAWDMWRAYSDMREANYIGA DKYFHARGNYDAAQRGPGGAWAAKVISDARENSQRVTD.FFRHGSSGHGA EDSKADQAAN~~~~~~~~~~~~~~~~~~~~~
abyssin cat ~~~~~~~~~~~~~~~~~~~~Q.WYSFLGEAAQGAWDMWRAYSDMREANYIGA DKYFHARGNYDAAQRGPGGAWAAKVISDARENSQRVTD.FFRHGNSGHGA EDSKADQXXXEWG~~~~~~~~~~~~~~~~~~
cat         ~~~~~~~~~~~~~~~~~~~~E.WYSFLGEAAQGAWDMWRAYSDMREANYIGA DKYFHARGNYDAAQRGPGGAWAAKVISDARENSQRVTD.FFRHGNSGHGA EDSKADQ...EWG~~~~~~~~~~~~~~~~~~
dsh cat     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MWXAYSDMREANYKGA DKYFHARGNYEAAQRGPGGAWAAKVISDARENSQRVTD.FFRSG~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
mouse1      MKLLTSLVFCSLLLGVCHGGFFSFVHEAFQGAGDMWRAYTDMKEANWKNS DKYFHARGNYDAAQRGPGGVWAAEKISDGREAFQEFF.........GRGH EDTIADQEANRHGRSGKDPNYYRPPGLPDKY
mouse CE/J  MKLLTSLVFCSLLLGVCHGGFFSFVHEAFLGAGDMWRAYTDMKEAGWKDG DKYFHARGNYDAAQRGPGGVWAAEKISDGREAFQEFF.........GRGH EDTMADQEANRHGRSGKDPNYYRPPGLPQKY
mouse2      MKLLTSLVFCSLLLGVCHGGFFSFIGEAFQGAGDMWRAYTDMKEAGWKDG DKYFHARGNYDAAQRGPGGVWAAEKISDARESFQEFF.........GRGH EDTMADQEANRHGRSGKDPNYYRPPGLPAKY
gdnhamster3a MKPFLAIIFCFLILGVDSQRWFQFMKEAGQGSTDMWRAYSDMREANWKNS DKYFHARGNYDAAKRGPGGAWAAKVISDAREGIQRFT.........GRGA ADSRADQFANKWGRSGKDPNHFRPAGLPSKY
hamster3    MKPFLAIIFCFLILGVDSQRWFQFMKEAGQGSTDMWRAYSDMREANWKNS DKYFHARGNYDAAKRGPGGAWAAKVISDAREGIQRFT.........GRGA ADSRADQFANKWGRSGKDPNHFRPAGLPSKY
gdnhamster3b MKPFLAIIFCFLILGVDSQRWFQFMKEAGQGSRDMWRAYSDMREANWKNS DKYFHARGNYDAAKRGPGGAWAAKVISDAREGIQRFT.........GRGA ADSRADQFANKWGRSGKDPHHFRPAGLPSKY
gdnhamster1 MKPFVAIIFCFLILGVDSQRWFQFMKEAGQGTRDMWRAYTDMREANWKNS DKYFHARGNYDAAQRGPGGAWAAKVISDAREGFKRIT.......GRGI EDSRADQFANEWGRSGKDPNFFRPPGLPSKY
gdnhamster2 MKPFLSIIFCFLVLGVDSQRWFQFMKEAGQGTRDMWRAYTDMREANWKNS DKYFHARGNYDAAQRGPGGAWAAKVISDAREGFKRMR.......GRGI EDSRADQFANEWGRSGKDPNFFRPPGLPSKY
mouse3      MRPSIAIILCILILGVDSQRWVQFMKEAGQGSRDMWRAYSDMKKANWKNS DKYFHARGNYDAARRGPGGAWAAKVISDAREAVQKFT.......GHGA EDSRADQFANEWGRSGKDPNHFRPAGLPKRY
rabbit2a    MKLLSGLLLCSLVLGVSGQGWFSFIGEAVRGAGDMWRAYTDMREANYINA DKYFHARGNYDAAQRGPGGVWAAKVISDVREDLQRLM.......GHGA EDSMDDQAANEWGRSGKDPNHFRPKGLPDKY
rabbit2b    MKLLSGLLLCSLVLGVSGQGWFSFIGEAVRGAGDMWRAYSDMREANYINA DKYFHARGNYDAAQRGPGGVWAAKVISDVREDLQRLM.......GHGA EDSMADQAANEWGRSGKDPNHFRPKGLPDKY
rabbit1     MKLLSGLLLCSLVLGVSGQRWFSFIGEATQGAWDMWRAYSDMREANYINA DKYFHARGNYDAAQRGPGGVWAAKVISDAREDLQRLM.......GHGA EDSMADQAANEWGRSGKDPNHFRPKGLPDKY
human1α     MKLLTGLVFCSLVLGVSSRSFFSFLGEAFDGARDMWRAYSDMREANYIGS DKYFHARGNYDAAKRGPGGVWAAEAISDARENIQRFF.......GHGA EDSLADQAANEWGRSGKDPNHFRPAGLPEKY
human1β     MKLLTGLVFCSLVLGVSSRSFFSFLGEAFDGARDMWRAYSDMREANYIGS DKYFHARGNYDAAKRGPGGAWAAEVISDARENIQRFF.......GHGA EDSLADQAANEWGRSGKDPNHFRPAGLPEKY
human2α     MKLLTGLVFCSLVLSVSSRSFFSFLGEAFDGARDMWRAYSDMREANYIGS DKYFHARGNYDAAKRGPGGAWAAEVISNARENIQRLT.......GHGA EDSLADQAANKWGRSGRDPNHFRPAGLPEKY
human2β     MKLLTGLVFCSLVLSVSSRSFFSFLGEAFDGARDMWRAYSDMREANYIGS DKYFHARGNYDAAKRGPGGAWAAEVISNARENIQRLT.......GRGA EDSLADQAANKWGRSGRDPNHFRPAGLPEKY
macaque     ~~~~~~~~~~~~~~~~~RSWFSFLGEAYDGARDMWRAYSDMKEANYKNS DKYFHARGNYDAAQRGPGGVWAAEVISDARENIQKLL.........GHGA EDT~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
rabbit3     MKLSIGIIFCFLILGVNSREWLTFLKEAGQGAKDMWRAYSDMREANYKNS DKYFHARGNYDAAKRGPGGVWAAEVISDARENYQKLI.........GRGA EDSKADQEANQWGRSGNDPNHFRPKGLPDKY
human3      MKLSTGIIFCSLVLGVSSQGWLTFLKAAGQGAKDMWRAYSDMREANYKKS DKYFHARGNYDAVQRGPGGVWATEVISDARENVQRLT.........GDHA EDSLAGQATNKWGQSGKDPNHFRPAGLPEKY
duck        ~~~~~~~~~~~~~DNPFTRGGRFVLDAAGGAWDMLRAYRDMREANHIGA DKYFHARGNYDAARRGPGGAWAARVISDARENWQ........GGVSGRGA EDTRADQEANAWGRNGGDPNRYRPPGLP
wallaby     MKLLTSLFLLSLVLCVNSQGWLRDSLDFLNQARLGAGDMWRAYRDMREANFKNS DKYFHARGNYDAAKRGPGGVWAAEVLSDAREFLQ........GGSSGRGV EDSMADQEANRWGRSGKDPNRYRPKGLDPKY
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Figure 1.3   SAA sequence allignment using the Wisconsin GCG Pileup program on the sequences in SwissProt database. Sequences are ordered by similarity. Abbreviations are: abyssin cat = Abyssinian cat; dsh cat = domestic short haired cat; gdnhamster = golden hamster. Human SAA allelic variations α and β are indicated. Other variant sequences from the same source name (rabbit SAA2 and golden hamster SAA3) are labelled a and b purely for ease of reference. The top two sequences are highly divergent and are separated by a dashed line to highlight this fact. Numbers at the top of the table mark the position of the first, fiftieth, and one hundredth amino acid using the first signal sequence amino acid as number one. Gaps in the sequences (. . . . .) are present to maximise homology.

of mink, dog, cat, cow and horse. Between residues 33 and 44 of the mature human SAA1 peptide is a region which is conserved in the SAA amino acid sequence of all species studied except murine and human SAA4 which both have four amino acid substitutions. In addition to the identified SAA alleles restriction fragment length polymorphisms have identified human SAA1 and SAA2 allelic variants, which differ at the nucleotide but not at the protein level. These protien variants have been designated as follows: SAA1 variants are SAA1α1, SAA1α2 and SAA1α3: SAA2 variants are SAA2α1 and SAA2α2 (Faulkes *et al* 1994, Faulkes *et al* 1997).

Although the secondary structure of SAA has not yet been reported some studies have used the amino acid sequence of SAA to predict the secondary structure (Turnell *et al*, 1986). The first 11 amino acids of the N-terminal constitute the most hydrophobic region of the sequence leading to the suggestion that it is this region which is involved in binding to HDL as discussed previously. Human SAA1 and SAA2 have a predicted amphipathic α-helical region between amino acids 8 and 32 at the N-terminal. A similar amphipathic region is present at the N-terminus end of SAA4 but between residues 1 and 12 and is hydrophilic compared to the other SAA's. A second α-helical region is present in SAA1 and SAA2 between residues 53 and 67, this region is also amphipathic. SAA4 differs here in that the corresponding region is a predominantly β-pleated sheet region and is much more hydrophobic. Similarly between residues 81 to 94 of SAA1 and SAA2 is a third α-helical region which is also amphipathic in both isoforms but is not found in SAA4. Residues 48-51 have been predicted to be a calcium binding site in SAA1. The octapeptide insert in SAA4 is a hydrophobic region which also contains a glycosylation site, approximately 50% of SAA4 molecules are glycosylated.

1.5.1.2. Functions of SAA

The physiological role of SAA has yet to be clearly elucidated. In order to define or discover a role for the protein consideration of the proteins characteristics or properties may yield some clues. The most notable of these properties and probably the most important when considering function are that SAA is an acute phase protein and that it exists predominantly as part of HDL_3.

A number of biological properties have been discovered for SAA. It has been found to have chemotactic properties (Badolato *et al* 1994). Recombinant human SAA (rSAA) has been shown to induce directional migration of monocytes and polymorphonuclear leucocytes. When rSAA was preincubated with HDL this chemotactic property was inhibited. This suggests that only free SAA and not the majority of SAA,which circulates as a part of HDL_3, is a chemoattractant. The same study also reported that rSAA regulated expression of the adhesion proteins CD11 and leucocyte cell adhesion molecule 1 and that when injected subcutaneously into mice it recruited polymorphonuclear (PMN) cells and monocytes at the site of injection. The same group subsequently reported evidence that recombinant human SAA can also induce directional migration of both sets of cells in vitro (Xu *et al* 1995). Inhibition of the migration of T cells by rSAA could be achieved by pretreating the cells with pertussis toxin indicating the involvement of a G-protein coupled receptor. In a similar to the previous study when mice were injected with human rSAA, T lymphocytes were found to be recruited to the site of injection.

There is additional evidence to that mentioned above for a role for SAA in cell adhesion. Preciado-Patt and coworkers (1996a) investigated the possibility that SAA may bind extra cellular matrix (ECM) components and examined the consequences of such binding. They discovered that rSAA could inhibit binding of T lymphocytes and melanoma cells to the

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extracellular matrix glycoproteins laminin and fibronectin. This appeared to be due to the presence of a cell adhesive motif associated with SAA. This led the group to investigate the possibility that SAA could in some way modulate adhesion of cells to the ECM. They found that binding of recombinant human SAA to components of the ECM is temporary but it does induce the adhesion of resting CD4+ cells to the ECM (Preciado-Patt *et al* 1996b) . Subsequently it was also found that recombinant human SAA also induces adhesion of mast cells to ECM or laminin. These investigation were carried out using recombinant human SAA and not SAA complexed with HDL_3 as it is predominantly found *in vivo*. However SAA complexed with HDL_3 as well as recombinant human SAA has been found to bind to human neutrophils.

Phospholipase A2 (PLA2) is an acute phase protein which is involved in the release of arachadonic acid from membrane phospholipids. There are two types of PLA2, a 97kDa cytosolic form and a 14kDa secreted form. Whereas cytosolic PLA2 non-selectively cleaves fatty acids from phospholipids, secreted PLA2 preferentially targets arachidonyl phospholipids. It has been found that although normal or non-acute phase HDL inhibits secretory non-pancreatic PLA2 activity, acute phase HDL (that is SAA enriched HDL) enhances the PLA2 activity (Pruzenski *et al* ,1995. The authors of the work related this finding to the role of HDL in reverse cholesterol transport speculating that SAA could modulate the flow of lipids as it had been reported that hepatic lipase, an enzyme with PLA2 like activity can hydrolyse HDL phospholipids to form HDL with increased ability to deliver cholesterol to cells.

As PLA2 causes the release of arachadonic acid which is then converted to active metabolites via the cycloxygenase and lipoxygenase pathways then it is possible that a protein that can enhance this enzyme could enhance the formation and hence activities of these metabolites. This possibity was examined by Malle and coworkers (1997) who found that human SAA1 was able to enhance the synthesis of cycloxygenase metabolites by activated monocytes

but not by resting monocytes. The mechanism by which this occurred however was not elucidated and so the authors were unable to confirm that modification of PLA2 activity was in fact involved.

SAA has also been found to inhibit oxidative bursts of neutrophils (Linke *et al*, 1991). Both SAA and acute phase sera were able to induce the oxidative burst of neutrophils in response to a bacterial fMLP peptide. In contrast normal and hence reduced SAA sera had a much reduced capacity to inhibit oxidative bursts. Inhibition of such bursts would aid in the prevention of tissue damage due to the release of reactive oxygen species.

It has been found that rabbit SAA3 can induce collagenase synthesis in rabbit synovial fibroblasts (Mitchell *et al* 1991). It was found that these cells, at a low passage number, produced relatively high levels of both SAA and collagenase. Anti-SAA3 IgG was able to prevent collagenase synthesis by the fibroblast cells. Synthesis of both the SAA and collagenase could be increased by the use of PMA or IL-1. This led the authors to suggest that fibroblasts may express rabbit SAA3 at sites of inflammation or injury and that the SAA3 acts in an autocrine fashion to increase collagenase synthesis.

SAA has been found to displace ApoAI from HDL suggesting a possible role in reverse cholesterol transport (Parcs and Rudell, 1985). As was mentioned previously there is an increase in the percentage of the total protein content of HDL_3 that is SAA, this must be at the expense of another protein or proteins. Apo AI is a co-factor for the enzyme LCAT (lecithin-cholesterol acyltransferase) which converts cholesterol to cholesterol ester, converting the HDL_3 to HDL_2, which is then transported back to the liver as part of chylomicron remnants and intermediate density lipoprotein (IDL) for excretion in the liver. The HDL_2 is then converted back to HDL_3 and can take up more cholesterol. The displacement of Apo AI by SAA results in inhibition of LCAT and logically this seems to imply that SAA would impair the transport

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of cholesterol from the extra hepatic tissues. This would coincide with reported findings of SAA in atherosclerotic lesions in which such accumulations are seen.(see section 1.5.1.3.3)

Lindhorst and colleagues (1997) put forward the hypothesis that the role of SAA is to remove cholesterol liberated from damaged cells at sites of injury. This theory suggests that instead of impairing the transport of cholesterol from the extra hepatic tissues, SAA would actually have the reverse effect removing extra hepatic cholesterol released from damaged cells and returning it to the liver. It stated that SAA modulated reverse cholesterol transport in order to target HDL to activated macrophages, which would be present at sites of injury or inflammation, where the SAA would be taken into the cells. Intracellular SAA influences the balance between cholesterol and cholesterol ester towards the former which is the more transportable form.

1.5.1.3. SAA and Disease

Although the physiological role of SAA has yet to be elucidated SAA has been implicated in the pathogenesis of particular disorders most notably the condition known as reactive amyloidosis. Most recently a number of reports have provided data which suggests there may be some evidence to link SAA to the formation of atherosclerotic plaques as SAA has been found to be expressed in these plaques.

1.5.1.3.1. Amyloidosis

Amyloidosis is a group of disorders, which can occur as a primary disease or secondary to another condition, and which are characterised by the presence of fibrous deposits in the organs and tissues.(table 1.2) The fibrous deposits can either be localised or systemic affecting a number of organs and tissues. They have a predominantly β-pleated sheet structure which is believed to be responsible for the positive staining found with congo red and which

gives an apple green birefringence under polarised light (Cooper, 1974). The major constituent of the fibrils can be one of 17 currently identified proteins (table 1.2). The fibres are formed from either the full length protein or from fragments of a precursor protein. The most common and well known form of amyloidosis is Alzheimer's Disease, a localised cerebral amyloid in which the major protein component is amyloid β protein and which is formed from Amyloid β Precursor Protein (Masters *et al*, 1985). N-terminal fragments of λ and κ Immunoglobulin light chain are the major constituent of the fibrils in AL amyloidosis which can occur as a primary disease or secondary to myeloma.

In addition to the fibrillar protein backbone the deposits also have two other major constituents common to all forms of Amyloidosis. These are serum amyloid P (SAP) protein (Baltz *et al*, 1986) and glycosaminoglycans (Nelson *et al*, 1991). SAP binds to the fibrillar components in a calcium dependent manor, although the ligand to which it binds is unknown, and constitutes 50% of the total mass of the amyloid deposits. Glycosaminoglycans are highly negatively charged complex carbohydrates which are extracellular matrix components. The glycosaminoglycans in amyloid deposits are derived from the connective tissue of the interstitium in which the amyloid fibrils are embedded. Other extracellular membrane components have also been found to be associated with amyloidosis fibrils, these are fibronectin, viteonectin and ubiquitin.

1.5.1.3.2. AA Amyloidosis

Amyloid A (AA) or reactive amyloidosis occurs secondary to conditions in which there is a prolonged acute phase response as would occur in chronic inflammatory conditions such as juvenile chronic arthritis. In these conditions there is prolonged elevated expression of the acute phase proteins including serum amyloid A. AA amyloidosis is a systemic amyloidosis with fibrous deposits being formed predominantly in the spleen, liver and kidney.

Amyloid Protein	Protein Precursor	Associated variant	Syndrome
AA	SAA (acute phase)		reactive: Juvenile Chronic Arthritis, familial mediteranian fever
AL	Immunoglobulin Light Chain		primary myeloma
AH	Immunoglobulin Heavy Chain		primary myeloma
ATTR	Transthyretin	Met 30 Met 111 TTR	familial amyloid polyneuropathy familial amyloidcardiomyopathy systemic senile amyloidosis
AApoAI	ApoAI	Arg 26	familial amyloid polyneuropathy aortic
AGel	Gelsolin	Asn 187ᶜ	familial amyloidosis
ACys	Cystatin C	Gln 68	hereditary cerebral haemorrhage
AFib	Fibrinogen α -chain	Val 526	hereditary renal amyloidosis
ALys	Lysozyme	Thr 56	Hereditary non-neuropathic amyloidosis
Aβ	Amyloid βprecursor protein		Alzheimers disease downs syndrome
Aβ2M	β_2-microglobulin		Dialysis
APrP	Prion Protein		spongiform encephalopathies
ACal	(Pro)calcitonin		medullary carcinome of the thyroid
AANF	Atrial Naturetic Factor		isolated atrial amyloid
AIAPP	Islet Amyloid Poly-peptide		islets of Langerhans, insulinomas
AIns	Insulin		primary aging pituitary prolactinomas
APro	Prolactin		

Table 1.2. The Amyloidosis disorders and associated proteins and precursors.

Serum amyloid A is the precursor of amyloid A which is the major constituent of the fibrils (Tape *et al*, 1988). Proteolytic cleavage of SAA in between Ser 76 and Leu 77 results in removal of the carboxy terminal end of SAA to leave the N terminal 76 amino acids which constitute Amyloid A protein. This cleavage may be necessary for the formation of Amyloid deposits however in some forms of amyloidosis it is the full length protein which forms the fibrils. Additionally a study by Kisilevsky and colleagues (1994) showed the presence of both the Carboxy and Amino terminus regions of SAA in amyloid deposits in the spleen indicating that the full length protein was present in the fibrils at some percentage.

AA Amyloidosis occurs when there is an elevated production of SAA. However impaired catabolism of the precursor protein SAA is believed to be a major factor in the pathogenesis of amyloidosis. Hebert and colleagues (1990) examined the uptake of SAA associated with HDL by splenic macrophages and Kupffer cells. They measured depletion of SAA-HDL from the media of cells over a 72 hour period and found a significant increase in the uptake of SAA by splenic macrophages from both normal and amyloidotic animals. In comparison no significant increase in uptake was observed with Kupffer cells. When serum amyloid P was added to the media it prevented this observed uptake by splenic macrophages indicating that Amyloid P protein could also be implicated in the pathogenesis of Amyloidosis.

Although AA amyloidosis is associated with chronic inflammatory conditions it does not occur in all patients in which these conditions exist. The prevalence of AA Amyloidosis in patients with systemic onset juvenile chronic arthritis (JCA) for example is approximately 10% . This suggests a genetic predisposition to amyloidosis. SAA has a number of family members and in some species amyloidosis is associated with a particular family member, in mice this is the isoform SAA2. In humans the homologue to mouse SAA2, SAA1 is the predominant isoform which is cleaved to form the fibrils. In addition to the multiple isoforms

of SAA there are also allelic variants of the protein.

The relative frequency of the three allelic variants of human SAA2 were examined in patients with juvenile chronic arthritis both with and without amyloidosis, and in healthy controls (Faulkes *et al*, 1997). A higher frequency of the SAA2α2 was found in the amyloidosis group compared to the JCA without amyloidosis group. Coinciding with this was a decrease in the frequency of the SAA2β allele compared with the non amyloidosis JCA group. When grouped together there was no significant difference in the allelic frequencies between the normal controls and the JCA group. Therefore this difference is related to amyloidosis and not to JCA. Recently it has been reported that SAA protein and mRNA expression has been found in the brains of patients with Alzheimers disease (Liang *et al*, 1997). Although Alzheimers disease is a form of amyloidosis it is associated with β amyloid protein not SAA. This is the first report to link SAA to the disease. However the presence of SAA in the brains of these patients is not evidence of involvement in the pathogenesis of the disease.

1.5.1.3.3. SAA and Atherosclerosis

There is evidence that SAA could be involved in the pathogenesis of atherosclerosis (Fyfe *et al*, 1997). Fatty streaks are the initial stage of atherosclerosis. The streaks are formed when monocytes and lymphocytes migrate between the arterial cells of the vessel wall, as they migrate the monocytes become macrophages and accumulate lipid to become 'foam' cells (Ross, 1993). The streaks progress to lesions when there is continued influx of cells to the site accompanied by migration of smooth muscle cells from the medial layer of the blood vessel to the intima. Here the smooth muscle cells proliferate and synthesise the components to form a connective tissue matrix composed of fibrous proteins, collagen and proteoglycans, this is followed by accumulation of extracellular and intracellular lipids and the resulting in the occlusive and potentially fatal atherosclerotic plaque.

In humans it has been found that there is a higher incidence of death due to cardiovascular disease in patients with rheumatoid arthritis than in age matched controls (Pincus *et al*, 1986). This provides evidence of a possible link between inflammation and atherosclerosis and the early pathophysiological processes that result in the formation of atherosclerotic plaques have been likened to inflammation. Further evidence linking inflammatory processes and atherosclerosis comes from findings that in mice fed an atherogenic diet there was induction of inflammatory genes (Liao *et al*, 1994., Liao *et al*, 1995). This finding was examined further and it was found that minimally modified LDL (mm-LDL), that is LDL that has been oxidised, was able to induce the same inflammatory genes as an atherogenic diet. Normal HDL can inhibit LDL oxidation however acute phase HDL which has a high SAA content was inefficient at preventing LDL oxidation. Acute phase SAA is infact pro-inflammatory (Van Lenten *et al*, 1995)

The implication of SAA in the pathogenesis of atherosclerosis leads to the question of whether it is hepatically produced SAA which is involved in the pathogenesis of the disease or whether SAA is being produced locally and it is this which is involved in atherosclerosis. SAA mRNA and protein expression has been reported in atherosclerotic plaques (Meek *et al*, 1994) and mRNA expression in smooth muscle cells (Kumon *et al*, 1997). In smooth muscle cells SAA expression can be induced by IL-1 but not by IL-6. The increase in IL-1 induced expression of SAA is mediated by the transcription factor NF-kB. NF-kB is also found to be upregulated in atherosclerotic plaques. This transcription factor is known to be upregulated in response to oxidative stress and hence mm-LDL is capable of inducing NF-κB. This occurs in the absence of *de novo* protein synthesis (see section 1.3.1.6.).

SAA expression has also been reported *in vitro* in monocyte/macrophage cell lines and smooth muscle cells (Ureli-Shoval *et al*, 1994.,Kumon *et al*, 1997). As has been previously mentioned

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SAA is believed to be involved in modifying reverse cholesterol transport and the N-terminal sequence (aa 1-11) is a lipid binding region. The ability of SAA to bind cholesterol and transport it into a number of cell lines was investigated by Liang and coworkers (1996). It was found that recombinant human SAA corresponding to SAA1 and a peptide corresponding to residues 1 to 18 of human SAA1 were able to bind cholesterol and facilitate its uptake into HepG2 cells and neonatal rabbit aortic smooth muscle cells. A peptide corresponding to human SAA1 from amino acids 14 to 44, human rSAA4 and a peptide corresponding to amino acids 1 to 18 of SAA4 did not facilitate uptake of cholesterol in HepG2 and smooth muscle cells or the other cell lines tested.

1.5.2. Serum Amyloid A gene family

1.5.2.1. Chromosomal Location of Human and Murine SAA

Four genetic loci for human SAA have so far been identified (Strachen *et al*, 1989., Betts *et al*, 1991). Two of these loci, SAA1 and SAA2, are polymorphic. The genes encoding the human serum amyloid A proteins have been mapped to a 100Kb region of chromosome 11 (fig 1.4.) (Sellar *et al*. 1994). SAA1 and SAA2 are located 18Kb apart and, as in the mouse , in opposite transcriptional orientations. SAA4 is located 11 Kb downstream of SAA2 and in the same transcriptional orientation. A human SAA pseudogene has also been identified and has been termed SAA3 it is located downstream of SAA4 but it's orientation is not known yet.

The genes encoding the murine serum amyloid A proteins have been mapped to a 79KB region of chromosome 7 (fig 1.4) (DeBeer *et al*, 1996). The genes encoding SAA1 and SAA2 are located approximately 8Kb apart and in opposite transcriptional orientations. A pseudogene (originally termed SAA4) has been identified 4Kb downstream of SAA1 (Lowel *et al* 1986).The SAA3 encoding gene is located 16 Kb downstream of this pseudogene

and in the same transcriptional orientation as SAA1. The SAA4 gene (originally termed SAA5) is located 1Kb downstream of the pseudo gene and 11 Kb upstream of SAA3.

1.5.2.2. Gene Structure and Homology

The SAA genes have a four exon structure, which is typical of a lipoprotein gene. SAA1 and SAA2 are highly homologous. In humans SAA1 and SAA2 are approximately 90% homologous over the entire coding region and extending to position -477bp of the 5' flanking region beyond this position the homology breaks down. SAA4 shares only about 55% DNA sequence homology with the other human acute phase genes. Similarly in mice SAA1 and SAA2 are 96% homologous and share a higher degree of homology with their human counterparts than with the mouse SAA3 or SAA4 genes. Evolutionary models have been proposed for both the mouse and human SAA gene families (fig 1.5). Both models show a common ancester for all the genes. For humans the common gene termed pre SAA duplicated to give the pseudogene SAA3 and the precursor to SAA1 and SAA2 known as pre SAA1/2 (Lowell *et al*, 1986, Yamamoto *et al*, 1987). This gene then duplicated to give SAA1 and SAA2. The similarity between these two genes particularly in certain regions suggests that in addition to being derived from a common gene that these genes have also undergone concerted evolution which due to the restricted area which is conserved and the breakdown of homology outside of this region suggests gene conversion. A similar model is observed with mouse with a common gene duplicating to give the pseudogene and a precursor to SAA1 and SAA2. These models however do not show the constitutive isoform or the mouse SAA3 which show the least degree of homology to the other SAA genes, so presumably originated from earlier duplication events.
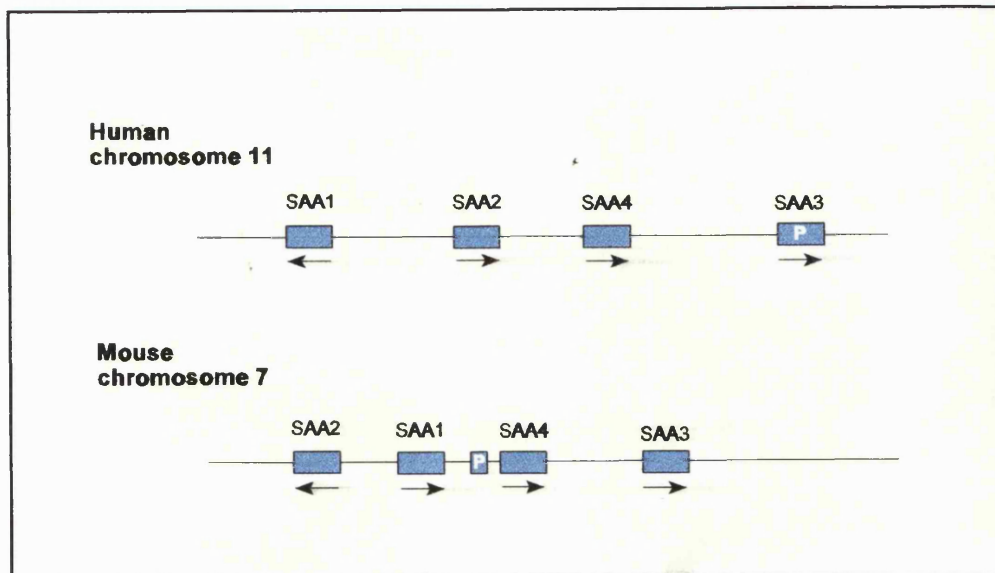
Figure 1.4. Chromosomal locatiion and orientaion of the human and mouse SAA genes families. P=pseudogene.

1.5.3. Transcriptional control of SAA

1.5.3.1. Cytokine induced expression

The acute phase nature of SAA dictates that in addition to the basic transcriptional machinery, control of expression of SAA must involve a mechanism which is responsive to conditions or substances produced during an acute phase response. Two main cytokine responsive elements are involved in the acute phase expression of SAA, these are cis-acting elements in the 5' flanking region of the acute phase SAA genes that bind the transcription factors NF-κB and C/EBP (fig 1.6) (Edbrooke *et al*, 1989., Betts *et al*, 1993).
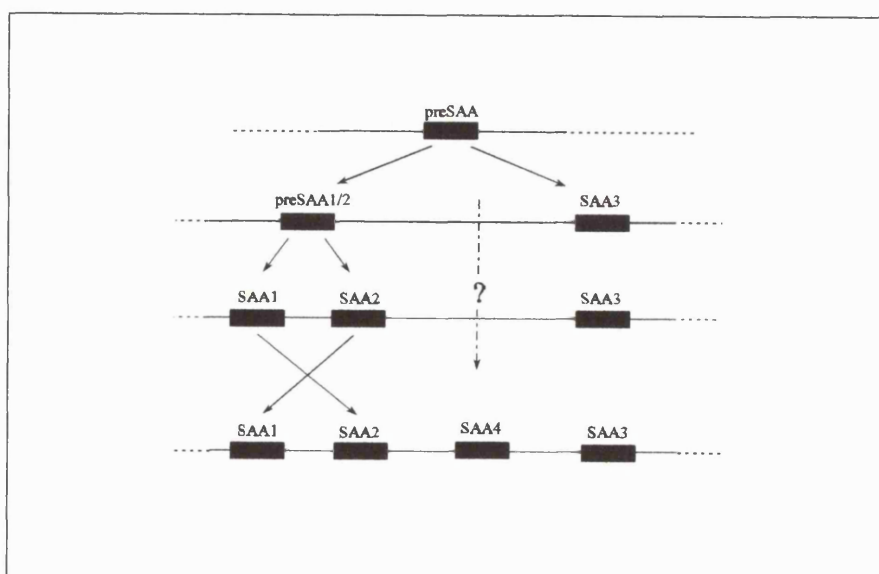
Figure 1.5. Schematic diagram of the proposed evolutionary development of the human SAA genes.

1.5.3.2. Human SAA expression and NF-κB

The involvement of NF-κB in SAA expression was originally identified due to the responsiveness of the gene to PMA. PMA was found to increase both SAA mRNA levels in cells and expression of SAA2' flanking region reporter constructs in transient transfection experiments (Edbroooke *et al*, 1989). A region which contained the consensus sequence for NF-κB was found to bind a nuclear factor both from PMA treated cells and, subsequently, cells treated with IL-1 (Edbrooke *et al*, 1991). This NF-κB site , at position -82 to -91, is one of two NF-κB sites within the SAA2 5' flanking region. The second ,at position -635 to -626, is part of a negative regulatory element (fig 1.7.). In transient transfection experiments this region appears to partly repress both constitutive and IL-1 induced expression although the fold increase in expression, due to IL-1, over constitutive levels remains the same (Edbrooke *et al*, 1991). This negative regulatory element was found to bind an IL-1 inducible factor thought to be of the NF-κB-like family. Immediately 5' to this site a constitutive factor binds to the SAA2 promoter, as shown by footprinting experiments . The negative regulatory region also contains the consensus sequence for C/EBP. The proximal NF-κB site (-82 to -91) is also present within the SAA1 gene due to the high degree of sequence homology within this region. The distal NF-κB site (-635bp to -626bp) is not present as the homology breaks down at -444 bp (HindIII site).

1.5.3.3. Human SAA Expression and C/EBP

An IL-6 responsive element has been identified within the SAA2 promoter at position -184 to -172. Originally identified as an NF-IL-6 binding site it has subsequently been found to contain the consensus sequence for, and bind factors of, the CAAT enhancer binding protein family (C/EBP). As with the proximal NF-κB site this site is also found within the SAA1 promoter due to the high degree of homology within this region.

```
        SAA1......AGCCTTGACCTCCTGGGTCTCCCACAATCCTTCCGCTTCAGCCTCTTGAG
-680              |  | |    | | | |   |                |   |   | |    ||
        SAA2......ACTCTCATTCTCCCAAGGTGGGGCTCCAGGACTAGGCTGGGGCAGCAGAA

        NFkB2
        TAGCTGGGACTGCAGGTGCACACTACCACACCCGGTCCATTTATTTTTATATTTCGTAGA
-631    |      || |   |    |    || ||   |      || |||||  ||||| |
        AGTCCCCCTCTCTACATTGTCCTTGGCTCAGGAGCCAACTTAGAAAAAGCATTTCCAAAT

        GACAAGATCGTTACAGTTTTGCACAGAGATCTTAAACTCTTGACCCCAAGTGATCCTCCT
-571         | |                |          |    |  | |   | |
        TGGCTAAGCCAGCGGAGCAGAGATTTTCTGTGCTGAGAAATATCAGGACATCCAGAGGGG

        GCCTTGGCCTCCAAAAGCATTGGGATTATAGGAGTGAGCCACTGTGCTGGACCTGTCAGC
-511      ||    |     ||      || | | | |   || |      |         | | |
        TGGAAGGAGGCTTCCAGGGCACACATGAGATGTGGCAGGGGTAGGCTGTCCGTTTTAAAG

            HindIII
        TTTGAAGCTTTAGATATGAACTCAGAGGGACTTCATTTCAGAGGCATCTGCCATGTGGCC
-451    ||  |||||||||| |||||||||| |||||||||   ||||  | ||||||||||||||||
        CTTAAAGCTTTAGACATGAACTCACAGGGACTTCA.GTCAGGGTCATCTGCCATGTGGCC

        CAGCAGAGCCCATCCTGAGGAAATGACTGGTAGAGTCAGGAGCTGGCTTCAAAGCTGCCC
-392    ||||||  |||||||||||||||||||||||| ||||  ||||||||||||||||  | |||||||||
        CAGCAGGGCCCATCCTGAGGAAATGACCGGTATAGTCAGGAGCTGGCTGAAGAGCTGCCC

        TCACTTCACACCTTCCAGCAGCCCAGGTGCCGCCATCACGGGGCTCCCACTCTCAACTCC
-332    |||||  ||||||||||||||||||||||||||||||||||||||||||||||||||  || |||
        TCACTCCACACCTTCCAGCAGCCCAGGTGCCGCCATCACGGGGCTCCCACTGGCATCTCT

        GCAGCCTCAGCCCCCTCAATGCTGAGGAGCAGAGCTGGTCTCCTGCCCTGACAGCTGCCA
-272    |||||  ||   |||  |||||||||||||||||||||| |||     ||||| |    |||||
        GCAGCTGCACTTCCCCCAATGCTGAGGAGCAGAGCTGATCTAGCACCCTGTCCATTGCCA

                                    C/EBP site
        GG..........CACATCTTGTTCCCTCAGGTTGCACAACTGGGATAAATGACCCGGGAT
-212    |           |        |||||||||||  |||||  ||||||||||||||||||||||||||||||||
        AGGCACAGCAAACCTCTCTTGTTCCCATAGGTTACACAACTGGGATAAATGACCCGGGAT

        GAAGAAACCACTGGCATCCAGGAACTTGTCTTAGACCGTTTTGTAGGGGAAATGACCTGC
-152    |||||||||||  ||||||||||||||||||||||||||||||   ||||||||||||||||||||||||
        GAAGAAACCACCGGCATCCAGGAACTTGTCTTAGACCAGTTTGTAGGGGAAATGACCTGC

        NFkB1 site
        AGGGACTTTCCCCAGGGACCACATCCAGCTTTTCTTCCCTCCCAAGAAACCAGCAGGGAA
-92     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||      ||
        AGGGACTTTCCCCAGGGACCACATCCAGCTTTTCTTCCCTCCCAAGAGACCAGC....AA

            TATA Box
        GGCTCAGTATAAATAGCAGCCACCGCTCCCTGGCAGGCAGGGACCCGCAGCTCA
-36     ||||||| ||||||||||||||||| |||||||||| |||||||||||||||||||||| Exon 1
        GGCTCACTATAAATAGCAGCCACCTCTCCCTGGCAGACAGGGACCCGCAGCTCA
```

Figure1.6   Comparison of the 5' regulatory regions of SAA1 and SAA2. Sites undelined: Exon1, TATA box, NF-κB1, C/EBP, HindIII site marking extent of homology, and NF-κB2. Numbering is with respect to the SAA2 sequence.

Figure 1.7. Transcriptional control elements involved in the constitutive and cytokine induced expression of human SAA2. C.F.= constitutive factor.

1.5.3.4. Transcriptional Control of SAA in Other Species

NF-κB and C/EBP are also the major components of transcriptional control of SAA in other non-human species. The rabbit SAA promoter contains two regulatory regions one binds members of the NF-κB family of proteins the other binds members of the C/EBP family of factors, these are C/EBPδ and C/EBPβ in the uninduced state and C/EBPδ when cells have been stimulated with LPS (Ray *et al* 1995). The rat SAA1 gene contains two C/EBP sites and one NF-κB site (Li and Liao, 1991).

Transcriptional control of the mouse SAA3 gene has been found to involve two separate elements (Huang et Liao, 1994). The proximal element contains two C/EBP sites and had tissue specific properties. The distal responsive element, which was cytokine responsive, consisted of two binding sites for members of the C/EBP family of proteins and one for

a novel constitutive factor. This constitutive nuclear factor was named SAA enhancer factor 1 (SEF-1). SEF-1 was found to be expressed in a number of cell types although at varying levels suggesting that it's main function is not regulation of tissue specific expression.

1.5.3.5. IL-1/IL-6 Synergistic Response of the SAA Gene

The response of the human SAA1 and SAA2 genes to IL-1 and IL-6 has been found to be synergistic. The increase in expression of the genes in response to both IL-1 and IL-6 is greater than the additive response of each cytokine separately. Xia and colleagues (1998) have shown that NFκB p65 and p50 but not p52 or C-rel could bind to the proximal NFκB site of the SAA2 promoter in response to IL-1 stimulation. Additionally C/EBPβ and C/EBPδ but not C/EBPα bind to the C/EBP site of the SAA2 promoter in response to IL-6 stimulation. The synergistic response to IL-1 and IL-6 observed for the SAA2 promoter could mean that NF-κB and C/EBP interact to form a transcription factor more effective than the combined effect of the individual factors. The ability of C/EBP to bind both full length p65 and mutants of the protein were examined. C/EBP was able to form a complex with p65 and mutants which contained the second half of the rel homology domain and the C-terminus of the activation domain. This indicates that it is these regions that are involved in the interaction of p65 with C/EBP. This synergism between Il-1 and IL-6 has also been found with other species. Expression of the rat and the rabbit SAA genes also appear to involve interaction between C/EBP and NF-κB factors (Li and Liao 1992, Ray *et al* 1995)

1.5.3.6. Repression of SAA Expression

A factor which actively represses expression of the rat SAA1 gene has been identified by Lu and coworkers (1994). They have identified a sequence within the rat SAA1 5' flanking region which overlaps the binding sites for NF-κB and C/EBP, that binds the factor ying-yang1 (YY1). YY1 binds to this region to actively repress SAA1 expression in the uninduced

state causing low basal levels of SAA1. Upon stimulation with conditioned media YY1 was displaced from it's binding site, NF-κB and C/EBP bind to their respective binding sites which were being blocked by YY1 and activate expression of the SAA1 gene.

1.5.3.7. Ying-yang 1 (YY1)

The transcription factor YY1 is a novel transcription factor in that it can repress, activate or initiate transcription. It has four zinc fingers belonging to the GLI-kruppel family which mediate DNA binding. The repressor and activator abilities of YY1 are attributable to distinct domains within the protein (Lee *et al* 1994., Lee *et al*, 1995., Bushmeyer et al, 1996). Genes known to be activated by YY1 include c-myc, IgH and HSV-1. Repression of transcription by YY1 is believed to be by more than one mechanism. The DNA binding site for YY1 can overlap the binding sites for other transcription factors, preventing DNA binding of the factor and hence preventing activation (Lee et al, 1992., Vincent et al, 1993.). The YY1 binding site has been found to overlap the binding sites for other transcription factors in a number of genes; In the regulation of the β-casein gene where YY1 overlaps a mammary gland factor site (Raught *et al*, 1994., Doppler *et al*, 1995); In the β-globin gene YY1 overlaps a GATA site (Raich *et al*, 1995)and as has already been discussed in the rat SAA1 gene YY1 overlaps a site for NF-κB (Lu *et al*, 1994). YY1 can also function through direct protein-protein interaction as occurs in the *c-fos* promoter by YY1-CREB interaction (Zhou *et al*, 1995)There is also evidence that YY1 repression involves bending of DNA to prevent interaction of transcription factors with the basic transcriptional machinery. By YY1 binding to the c-fos promoter YY1 induces a 80° bend in the DNA(Natesan *et al*, 1993).

1.5.4. Tissue Specific Expression of SAA

As with other acute phase proteins SAA is predominantly produced by the liver (Kushner, 1982). A cell-specific element has been identified within the mouse SAA3 promoter which appears to confer the high hepatic expression to SAA (Li *et al*, 1990).The element ,located at positions -63 to -93bp of the 5' flanking region, was capable of conferring liver cell specific expression to a heterologous promoter. The element was found to contain two binding sites for the C/EBP family of proteins. Other than this, mechanisms for SAA tissue specificity have not been analysed in depth.

1.5.4.1. Serum Amyloid A Tissue Distribution

Although the major site of SAA production is the liver, extra hepatic expression of SAA has also been reported (Meek and Benditt, 1986 and Ramadori *et al*, 1985). Meek and Benditt examined SAA expression in 14 murine tissues after casein or LPS injection and found that although casein induced SAA expression in the adrenal glands only , LPS induced expression in all tissues tested which included kidney, liver and intestine. Examining the expression of each isoform separately they found different patterns of expression for each isoform. While kidney expressed all three isoforms at relatively high levels the predominant extra-hepatically expressed isoform was SAA3.

The same group also examined SAA expression in different rat tissues in response to LPS and turpentine (Meek and Benditt 1989). It was found that SAA mRNA was expressed in rat ileum and large intestine without stimulation and expression was not significantly increased by LPS or turpentine injection. Liver expressed SAA after injection with both LPS and turpentine, lung expressed SAA in response to turpentine but not LPS whereas kidney and adrenal gland were found to express SAA in response to LPS but not turpentine. In the liver two different size mRNAs were found to be produced in response to turpentine

whereas only one of these was produced in response to LPS. When these mRNAs were examined it was found that the mRNAs produced in liver were related to mouse SAA1 and SAA2 whereas the extrahepatically produced SAA is related to mouse SAA3.

Extra hepatic expression of human SAA has also been detected. The macrophage precursor cell line THP-1 has been found to express SAA mRNAs in response to dexamethasone alone and dexamethasone with LPS, IL-1, IL-6 and IL1 & IL-6 after differentiation and additionally in response to dexamethasone with LPS without differentiation (Ureli-Shoval *et al*, 1994). Further analysis of the mRNAs found that SAA1, SAA2 and SAA4 were all being expressed.

Steel and associates (1996) examined the expression of both acute phase and constitutive SAA in a number of hepatic and non-hepatic cell lines.Using northern blot analysis, and without induction of the cells, they found two different sized bands corresponding to constitutive SAA in hepatic cell lines. One of these bands was also present for all the non-hepatic cells tested. Extra-hepatic expression of acute phase SAA was also found. Using monocyte conditioned media acute phase SAA expression could be induced in the non-hepatic cell lines KB (oral epidermal carcinoma) and also at a low level in RT4/31 (bladder papilloma).

1.5.4.2. SAA Activating Sequences (SAS) and SAS Activating Factor (SAF)

An element responsible for induced expression of rabbit SAA3 in non-hepatic cells has been identified (Ray and Ray 1996). The element, located between - 280 and -224 of the 5' flanking region, contains three homologous sequence motifs which have been called SAA activating sequence (SAS) and binds a 55kDa protein. Although the element was found to confer IL-6 responsiveness in both hepatic cells and non-hepatic cells (lung fibroblasts and synoviocytes)

absence or mutation only prevented IL-6 induced SAA expression in the non-hepatic cells. IL-6 was still able to induce SAA expression in hepatic cells, suggesting other mechanisms are involved in this process. Sequence analysis of the binding site revealed no homologies to known transcription factors and antibodies to a number of known cytokine induced transcription factors were unable to super shift the protein DNA complex in electrophoretic mobility shift assays. This indicated that this was a novel protein and it was termed SAF (SAS activating factor). Subsequent studies by the same group have shown that SAF is present in human THP-1 cells and can bind to and activate rabbit SAA3 reporter constructs in response to LPS(Ray and Ray, 1997., Ray and Ray, 1997b).

1.6. Mechanisms of Liver Specific Expression

The study of a number of genes predominantly expressed in the liver has shown that liver specific expression involves a number of transcription factors which are predominantly but not exclusively expressed in the liver (reviewed in Grayson *et al*, 1989; Lai and Darnell, 1992; DeSimone and Cortese, 1992). These factors are C/EBP, The HNF-1 family, the HNF-3 family, HNF-4, HNF-5 (Grange *et al*, 1991) and HNF-6 (Smadani and Costa, 1996). Although each of these factors is expressed in a wide range of extrahepatic tissues the only tissue in which they are all found is the liver . Liver specific genes have been found to contain binding sites for multiple liver enriched transcription factors which, in combination, mediate hepatic specific expression. Thus the term 'liver specific expression' is often a misnomer as it refers to liver dominant expression.

1.6.1. C/EBP

The C/EBPs are a family of transcription factors C/EBPα , C/EBP β, C/EBPγ , C/EBPδ and C/EBPε have been so far identified. The C/EBP family of factors all bind to the same DNA sequence as either homodimers or heterodimers with other C/EBPs. They all contain

a leucine zipper motif through which they dimerise. The different C/EBPs exhibit different tissue patterns of expression. C/EBPα is predominantly expressed in liver and adipose tissue, but is also found in other tissues (Xanthopoulos *et al* 1989,Antonson and Xanthopoulos 1995). C/EBPβ is also expressed in a number of tissues but is predominantly found in the liver and kidney (Cao *et al* 1991). C/EBPγ is ubiquitously expressed (Roman *et al*). C/EBPδ is predominantly expressed in lung although as with the other isoforms expression is also found in other tissues (Cao *et al* 1991). C/EBPε expression appears to be limited to myeloid and lymphoid cells .

1.6.2. HNF-1

HNF-1 was originally identified as a factor which bound the promoter of the α-1 antitrypsin gene (DeSimone *et al*, 1987) and was involved in the liver specific expression of the gene, since then HNF-1 binding has been observed in a number of liver specific genes. HNF-1 is a highly conserved protein in mammals with 94% homology between the rat and human proteins (Wach et al, 1991). HNF-1 must dimerise to bind DNA(Chouard et al, 1990). In addition to homodimerisation HNF-1 can form heterodimers with another liver enriched protein which was found to bind the same DNA sequence as HNF-1 and was termed vHNF (Rey-Campos et al, 1991., Wach et al, 1991). These factors have subsequently been renamed HNF-1α (HNF-1) and HNF-1β (vHNF-1). HNF-1 has a dimerisation domain within its amino terminal region and two DNA binding domains both of which are required for the factor to bind to DNA. One of these binding domains is a homeodomain the other a POU domain. HNF-1α also has a transactivation domain at amino acids 281 to 628. HNF-1β is highly homologous to HNF-1α in the dimerisation and DNA binding domains but not in the activation domain. A protein termed DCoH has been reported to stabilise the HNF-1 dimers and is expressed in the same tissues as the factors (Mendel *et al*, 1991). HNF-1α expression is not restricted to the liver it is also found in kidney intestine and pancreas as

is HNF-1β albeit at different levels (Blumefeld et al, 1991., De Simone et al, 1991., Lazzaro et al, 1992).

1.6.3. HNF-3

HNF-3 was identified as a factor which bound to, and was involved in, liver specific transcription of the transthyretin gene (Costa et al, 1990). There were subsequently found to be three members of the HNF-3 family HNF-3α, HNF-3β, and HNF-3γ (Mendel et al, 1991). The binding domains of the three HNF-3 factors are highly homologous (approx 85% homology). These three factors, like the HNF-1 proteins, are expressed in other tissues in addition to the liver. They have been found to be expressed in tissues derived from the lining of the primitive gut. All three isoforms are found in liver and intestine, HNF-3α and HNF-3β are also expressed in lung and HNF-3γ in testis.

1.6.4. HNF-4

HNF-4 was found to bind regulatory regions within the transthyretin and apolipoprotein CII promoters (Mietus et al, 1992.). HNF-4 is a member of the steroid receptor superfamily. The DNA binding domain is located at amino acid 50 to 133 and has a zinc finger structure. Amino acids 133 to 373 constitutes the ligand binding domain. HNF-4 also binds to DNA as a dimer. It is expressed in kidney and intestine as well as liver (Costa et al, 1989).

1.6.5. HNF-5 and HNF-6

HNF-5 and HNF-6 are the most recently identified of the liver specific transcription factors. HNF-5 was identified in glucocorticoid responsive units of the rat tyrosine aminotransferase gene and has also been implicated in the tissue specific expression of a number of other genes (Grange et al 1991, Shachter et al 1993). HNF-6 was identified as a factor involved

in the liver specific expression of the HNF-3β gene (Samadani and Costa, 1996). It was also found to bind a site in the transthyretin promoter which was originally identified as a HNF-3 site.

1.6.6. Positive and Negative Feedback Regulation of Liver Enriched Factors

HNF-4 has been found to positively regulate HNF-1α and is necessary for its expression (Kuo et al, 1992). HNF-1 does not regulate HNF-4 but does negatively regulate itself. HNF-3 is also an autoregulatory protein having a negative regulatory effect on its own expression as does C/EBP.

1.6.7. Other Liver Specific Genes

The liver specific expression of the α1-antitrypsin and transthyretin genes ,both of which are acute phase proteins, has been shown to involve different combinations of the factors C/EBP, HNF-1, HNF-3 and HNF-4 (Costa *et al*, 1990., De Simone *et al*, 1992) (fig 1.8). The albumin gene contains multiple binding sites for C/EBP and an HNF-1 binding site in addition this the region contains a binding site for the ubiquitous factor NFY (Cereghini *et al*, 1988).
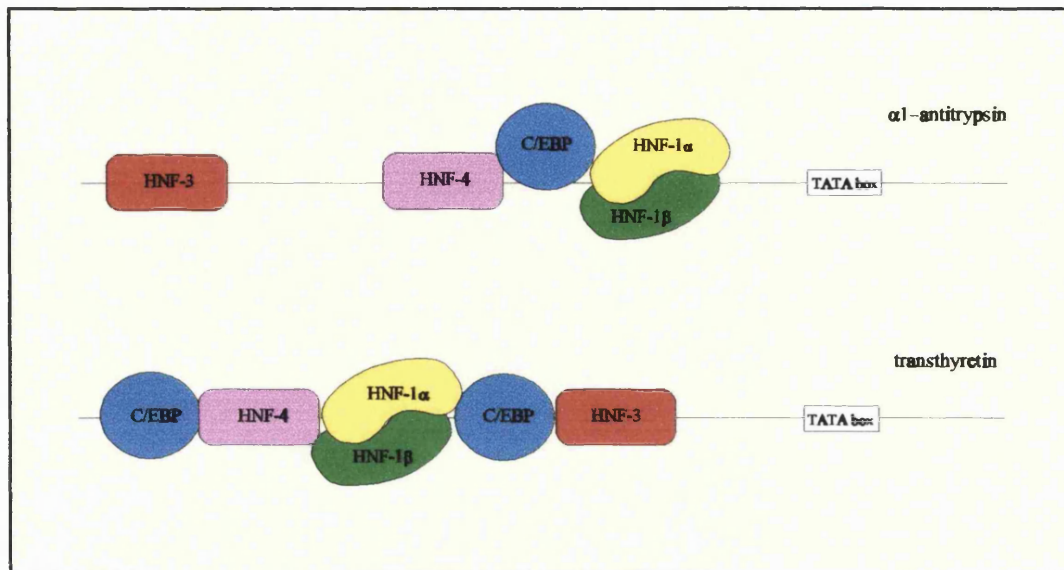
Figure 1.8. Transcription factors involved in the liver specific expression of the α-1antitrypsin and transthyretin genes

1.7. Aims of the Project

The aim of the project was to examine the tissue specific expression of SAA. Initially the tissue distribution of SAA expression would need to be examined in order to confirm that SAA does exhibit tissue specific properties. In the light of recent reports of extra hepatic expression of SAA it can no longer be taken for granted that expression is liver specific. If SAA is found to be exclusively or predominatly expressed in the liver then this project would aim to identify the elements mediating this tissue specificity. Initially this would be by use of SAA2 5' flanking region reporter constructs transfected into hepatic and non-hepatic cell lines. The outcome of these transfection experiments would allow for sequence analysis of specific regions of the SAA2 gene. It would also enable specific regions of the gene to be analysed for binding of nuclear proteins. If such DNA protein complexes are formed then the proteins involved would be identified by the use of antibodies to candidate proteins (such as the liver enriched factors) and by DNA footprinting.

2. Materials

2.1. Probing of multiple tissue mRNA dot blot reagents

50 x denharts solution -

      5g ficoll

      5g polyvinylpyrolidine

      5g bovine serum albumin

20 x SSC -

      175.3g NaCl

      88.2g NaCitrate

      to 1l with dH_2O

      pH to 7.0

herring sperm - 10mg/ml dH_2O, Sheared using an 18 gauge needle and boiled for 10 mins

prehybridisation soln -

      6 x SSC

      5 x denharts solution

      0.1% SDS

      150μg /ml denatured sheared herring sperm

hybridisation soln -

      6 x SSC

      5 x denharts solution

      150μg/ml sheared denatured herring sperm

2.2. Cell Culture reagents

Dulbeccos modified essential media (DMEM)

supplemented with -

      10% bovine feotal calf serum

2mM glutamine

100 IU/ml penicillin

100μg/ml streptomycin

non-essential amino acids

2.3. PCR reagents

10 x PCR buffer -

100mM tris pH 8.4

500mM KCl

25mM $MgCl_2$

2mM dNTP's

2.4. Preperation of plasmid DNA - media and reagents
2.4.1 Media components

LB (Luria-Bertani) medium -

10g bacto-tryptone

5g bacto-yeast

10g NaCl

make up to 1l with dH_2O and pH to 7.0

Terrific broth -

48g EzMix terrific broth base

8 mls glycerol

to 1 liter with dH_2O

SOC medium -

20g bactotryptone

5g bacto-yeast extract

10g NaCl

make up to 1l with dH_2O and pH to 7.5

proir to use add 1/100th volume of 1M $MgSO_4$, 1M $MgCl_2$, and 2M Glucose.

agar plates -

1.5g agar per 100 mls LB media

Ampicillin -

      100mg/ml in dH$_2$O , filter sterilised

      used at 100ng/ml final concentration

2.4.2. Small scale preperation of plasmid DNA

      Quiaprep spin miniprep kit from Quiagen

2.4.3. Large scale preperation of plasmid DNA

soln I -

      50 mM glucose

      25mM Tris pH 8.0

      10mM EDTA pH 8.0

      sterilised by autoclaving and stored at 4°C

soln II-

      1% SDS

      0.2 M NaOH

      made fresh

soln III-

      180 mls 5M KAc

      34.5 mls glacial acetic acid

      make to 300 mls with dH$_2$0, make fresh

T.E. -

      10mM Tris-HCL pH 8.0

      1mM EDTA pH 8.0

2.5. Transient transfection reagents

Hepes buffered saline (10 x)

      8.18% Nacl

      5.94% HEPES

      0.20% Na$_2$HPO$_4$

      filter sterilised

T.E. (equivalent to a 1:10 dilution of standard T.E.)

    1mM tris-HCl pH 8.0

    0.1 mM EDTA

2.6. Reporter construct assay reagents

luciferase assay substrate -

    20 mM Tricine

    1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$

    2.67 mM $MgSO_4$

    0.1mM EDTA

    33.3 mM DTT

    270 μM Coenzyme A

    470 μM luciferin

    530 μM ATP

compositions of other reporter assay reagents were not available from the manufacturers.

2.7. Gel electrophoresis reagents

50 x TAE buffer -

    242g Tris base

    57.1mls glacial acetic acid

    100 mls 0.5M EDTA (pH 8.0)

    to 1l with dH_2O

10 x TBE buffer -

    108g tris base

    55g boric acid

    40 mls 0.5M EDTA (pH 8.0)

Ethidium bromide -

    10mg/ml stock solution with dH_2O

    used at a final concentration of 300-350ng/ml molten agarose

Agarose gels - 1% agarose in 1 x TAE buffer, run in 1 x TAE buffer

polyacrylamide gels -

% acrylamide gel	5%	8%
40% acrylamide*	6.25mls	8.0 mls
10 x TBE buffer	5.0 mls	5.0 mls
ammonium persulphate	0.1g	0.1g
TMED	30 µl	30 mls
1M DTT	10 µl	10 ul
dH$_2$O	to 50 mls	to 50 mls

      run in 0.5 x TBE buffer

* acrylamide:bisacrylamide 19:1

Sequencing and footprinting gels

      10 mls 40% acrylamide:bisacrylamide 19:1

      25g urea

      5 mls 10 x TBE

      15 mls dH$_2$O

      when dissolved added

      350µl fresh 10% ammonium persulphate

      35µl TEMED

      run in 0.5 x TBE buffer

10 x Loading buffer -

      0.25% bromophenol blue

      0.25% xylene cyanol FF

      15% Ficoll

2.8. Nuclear extract preperation reagents

buffer A -

      10 mM Hepes pH 7.8

      1.5 mM MgCl$_2$

      60 mM KCl

      10 mM NaCl

      0.5 mM EDTA

      0.5 mM DTT

buffer C -

      40 mM Hepes pH 7.8

      50 % glycerol

      3mM $MgCl_2$

      0.5 mM EDTA

protease inhibitors (added to both buffer nuclear extract buffer A and buffer C) -

      phenylmethylsulfonyl fluoride (PMSF)

      100mM stock soln in isoprpanol used at a working concetration of 0.2mM

      complete protease inhibitor tablets EDTA free

      working concentration 1 tablet per 25 mls buffer

2.9. protein binding studies reagents

poly dI-dC - 6µg/µl stock solution in 1mM EDTA

                 40mM KCl

                 5mM $MgCl_2$

                 2.5 mM DTT

                 heated at 65°C for 5 mins

2 x parker buffer

      16% Ficoll

      40 mM Hepes pH 7.9

      100 mM KCl

      2 mM EDTA pH 8.0

      1 mM DTT

2.10. Suppliers

Restriction and Modification Enzymes   New England Biolabs, MA, U.S.A.

BsRDI restriction enzyme   Promega, Madison, WI, U.S.A

All other Restriction enzymes   "

Taq DNA polymerase   "

T4 DNA Ligase   "

Klenow fragment of DNA polymerase I

Radionucleotides   Amersham International, Amersham, U.K.

$[\gamma\text{-}^{32}P]ATP$ (3000 Ci mmol^{-1})   "

$[\alpha\text{-}^{32}P]dCTP$ (3000 Ci mmol^{-1})   "

$[\alpha\text{-}^{35}S]dATP$ (100 Ci mmol^{-1})

Cytokines   Genzyme Corporation, Boston, MA, U.S.A.

IL-1β

IL-6

Cell Culture Media   GibcoBRL Life Technologies, Gaithersburg,

Dulbeccos modified essential Media   MD, U.S.A.

Bovine feotal calf serum

non-essential amino acids

penicillin

streptomycin

Hanks balanced salt solution

Trypsin-EDTA   Boerhringer Mannheim, Lewes Sussex,U.K.

complete protease inhibitor tablets

      Qiagen, Crawley, Surrey, U.K.

Quiaspin miniprep kit

Quiex gel isolation kit

Thermocycle sequencing kit   New England Biolabs, MA.U.S.A

3 Methods

3.1 Dot Blot Analysis

A multiple human tissue mRNA dot blot was obtained from Clonetech. SAA oligonucleotides (fig.3.1) and a ubiquitin cDNA fragment were used as probes. Oligonucleotides used were pw242 which has a Tm of 66 corresponding to region within exon 3 of human SAA1 and SAA2, pw 241 corresponding to the region of human SAA4 which contains the octapeptide insert and a human ubiquitin cDNA probe (provided with the blot)

```
SAA1  CCTACTCTGACATGAGAGAAGCCAATTACATCGGCTCAGACAAATACTTC
SAA2  CCTACTCTGACATGAGAGAAGCCAATTACATCGGCTCAGACAAATACTTC
          pw 242 AGCCAATTACATCGGCTCAGAC


SAA1  AGATTCTTTG........................GCCATGGTGCGGAGGA
SAA2  AGACTCACAG........................GCCGTGGTGCGGAGGA
SAA4  GGATTAATAGACTACTATTTATTTGGAAACAGCAGCACTGTATTGGAGGA
          pw 241 AGACTACTATTTATTTGGAAACAGCA
```

Fig 3.1 Human SAA1/SAA2 specific and SAA4 specific oligonuleotides used to detect SAA mRNA species on a multiple human tissue mRNA dot blot.

3.1.1 Radiolabelling of oligonucleotide probes for use in dot blot analysis.

Oligonucleotides for use as probes were end labelled with γ-ATP ^{32}P. A 25 ng quantity of single stranded oligonucleotide of 20-30 bp in length was incubated in a reaction

containing 70mM Tris-HCl pH7.6, 10mM $MgCl_2$, 5mM DTT, 50 μC ATP ^{32}P and 1 unit

of bacteriophage T4 polynucleotide kinase in a total reaction volume of 10 μl. (The Curie

unit was originally defined as the quantity of ^{222}Rn, a decay product of ^{226}Ra, in which the

number of atoms disintegrating per second was equal to 1g of ^{226}Ra. This has been

extended to include all radioactive materials and one Curie is now defined as the quantity

of radioactive material in which the number of atoms disintegrating per second is 3.7 x

10^{10}.) In the presence of excess ADP bacteriophage T4 polynucleotide kinase catalyses

the transfer of the 5' terminal phosphate from DNA (or RNA) to ADP, and the subsequent

rephosphorylation of the DNA by transfer of the γ phosphate group from γATP P^{32}

(Richardson , 1965,. Richardson, 1971). Synthetic oligonucleotides lack the terminal 5'

phosphate group and T4 polynucleotide kinase can catalyse the transfer of the γ

phosphate group of γATP P^{32} in the absence of ATP (Agarwal *et al*, 1979).

Unincorporated γ-ATP ^{32}P was removed using -10 chromaspin columns (Clonetech).

These columns are designed to bind and retain unincorporated nucleotides and allow the

DNA to pass through. The size of DNA will be eluted through is 10bp or greater hence

the columns are designated -10. Columns are available cut off points for larger DNA

fragments. The reaction mixture was made up to 50 μl with dH_2O, the column was

prespun to remove the buffer from the column. The end labelling reaction mix was then

added to the resin on the column and the column spun at 700xg for 5 mins. The

unincorporated nucleotides remain on the column and the labelled oligonucleotide passes

through.

3.1.2 Radiolabelling of cDNA ubiquitin probe

Ubiqutin cDNA was used as the control.This was radiolabelled by random primer labelling

using the ready to go labelling kit from Pharmacia Biotech. The radioisotope used was α-

P^{32} dCTP.

3.1.3. Probing of human multiple tissue dot blot

The dot blot was incubated in a prehybridisation solution (for recipe see section 2.1) for 30 mins at 36°C for the oligonucleotide probes and 65°C for the cDNA probe with constant agitation in a Techne HD-1B hybridisation oven. The prehybridisation solution was replaced with hybridisation solution (for recipe see section 2.1) to which was added the radiolabelled oligonucleotide probe which had been denatured at 95°C for 5 mins then chilled on ice. Hybridisation was carried out at the same temperature as prehybridisation with constant agitation for 16 hours. The hybridisation solution and probe were removed and the blot washed 4 times in a solution containing 2x SSC and 1% SDS for 20 mins at room temp followed by one wash at the hybridisation temp. Autoradiography using kodak x-omat film was performed at -70°C.

3.1.4. Densitometry analysis of dot blot data

Exposed x-ray films were analysed by densitometry analysis using the ampligene imager system and accompanying densitometry software. The autoradiographs are photographed using the ampligene system camera and the image saved to floppy disc. The image is analysed using the ampligene 1D and 2D densitometry software. The region to be analysed is outlined and 'spot' analysis carried which automatically detects the dark regions or 'spots' on the autoradiography and determines the area and optical density of each one.

3.1.5. Removal of oligonucleotide probes

Oligonucleotide probes were removed from the dot blot by incubating the blot in a solution containing 0.5% SDS, heated to 100°C, for 10 mins with constant agitation. The procedure was repeated once.

3.2 Construction of luciferase reporter constructs

The pGL2-basic luciferase reporter vector (fig 3.2.) was used to make reporter constructs to analyse the SAA2 5' flanking region. The pGL2-basic vector is 5597 kb in size, it contains the cDNA encoding firefly luciferase, an ampicillin resistance gene for use in screening of positive clones, an f1 origin of replication derived from filamentous phage, an origin of replication in E.coli (ori) an SV40 region containing a poly(A) signal which lowers background activity of the promoter from spurious transcription of the reporter gene. The vector contains a multi cloning site with 8 restriction enzyme sites for use in the cloning of DNA fragments in to the reporter vector .
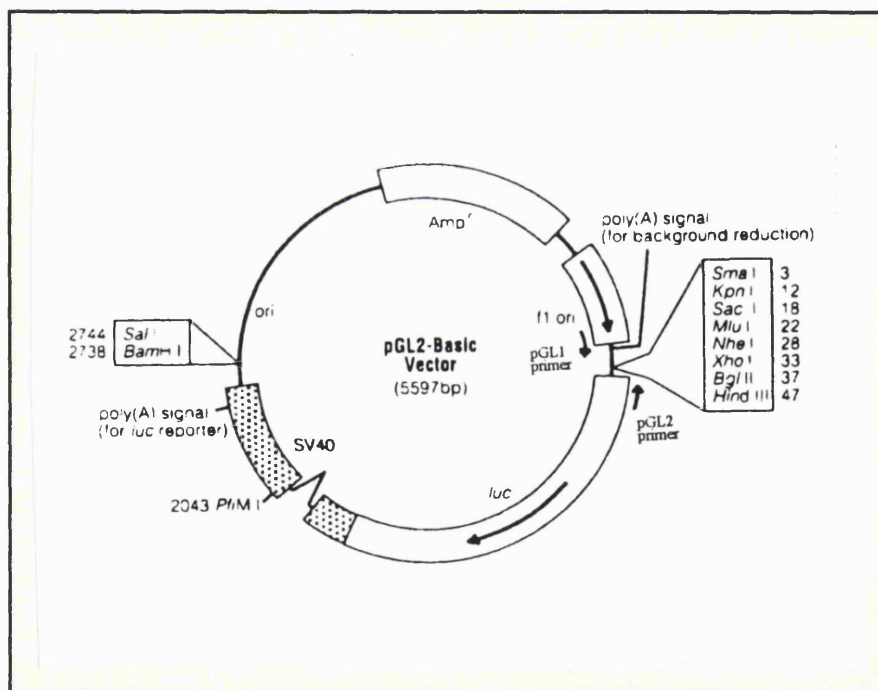
Fig 3.2. The pGL2 luciferase reporter vector used to make SAA2 5' flanking region reporter constructs.

DNA fragments containing regulatory elements which are cloned into the polylinker drives expression of the reporter gene, the cDNA for firefly luciferase. Luciferin is a organic acid present in fireflies, it reacts with ATP to form the intermediate luciferyl-adenylate. In the presence of oxygen and magnesium the enzyme luciferase catalyses the oxidation of the intermediate producing oxy-luciferin, ADP and a photon of light (DeWet et al, 1987) (fig 3.3.). Relative levels of expression of the gene, due either to the vector promoter or inserted DNA fragments, are determined by measuring the ability of the luciferase to catalyse this reaction.

luciferin + ATP $\longrightarrow$ luciferyl-AMP $\xrightarrow{\text{luciferase}}$ Oxy-luciferin + ADP + light
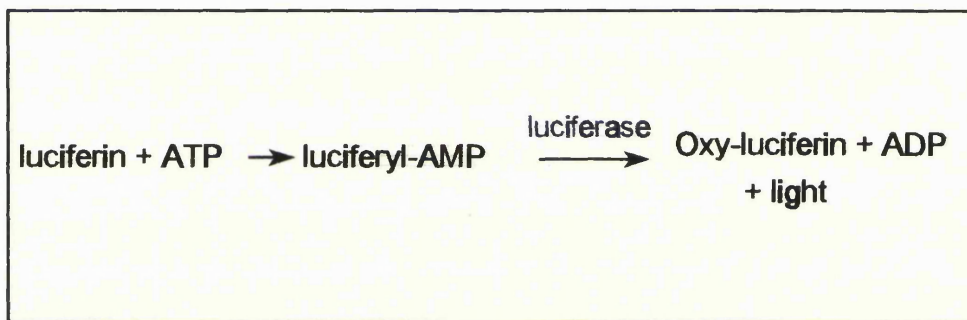
Fig. 3.3. The light emiting reaction which occurs in the firefly and is catalysed by the enzyme luciferase

Fragments of the SAA2 5' flanking region were used to make the reporter constructs (fig 3.4). The fragments were identical to those used in chloramphenicol acetyl transferase (CAT) reporter constructs by Edbrooke and collegues previously in this laboratory (Edbrooke et al, 1991). The Bam-luc construct was made by digestion of the Bam-CAT construct with BamHI and BglII to remove the fragment from the CAT reporter construct. The fragment was then ligated into the bglII site of the pGL2 polylinker region. The Xmn-luc construct was made by digesting Bam-CAT with XmnI and BglII and cloning the isolated fragment into the SmaI and BglII sites of the pGL2-Basic vector. The Sst-luc construct was made by digesting Sst-CAT with SstI and BglII and cloning the isolated fragment into the SmaI and BglII sites of the pGL2-Basic vector. The Hind-luc construct was made by digesting the Bam-luc construct with HindIII to remove the region

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from the -444 (HindIII site) to the hindIII site of the multicloning site of the plasmid. The fragment was cloned into the HindIII site of the pGL2-basic vector. The Sau-luc construct was made by digestion of the Sau-CAT construct (Edbrooke *et al*) with SalI
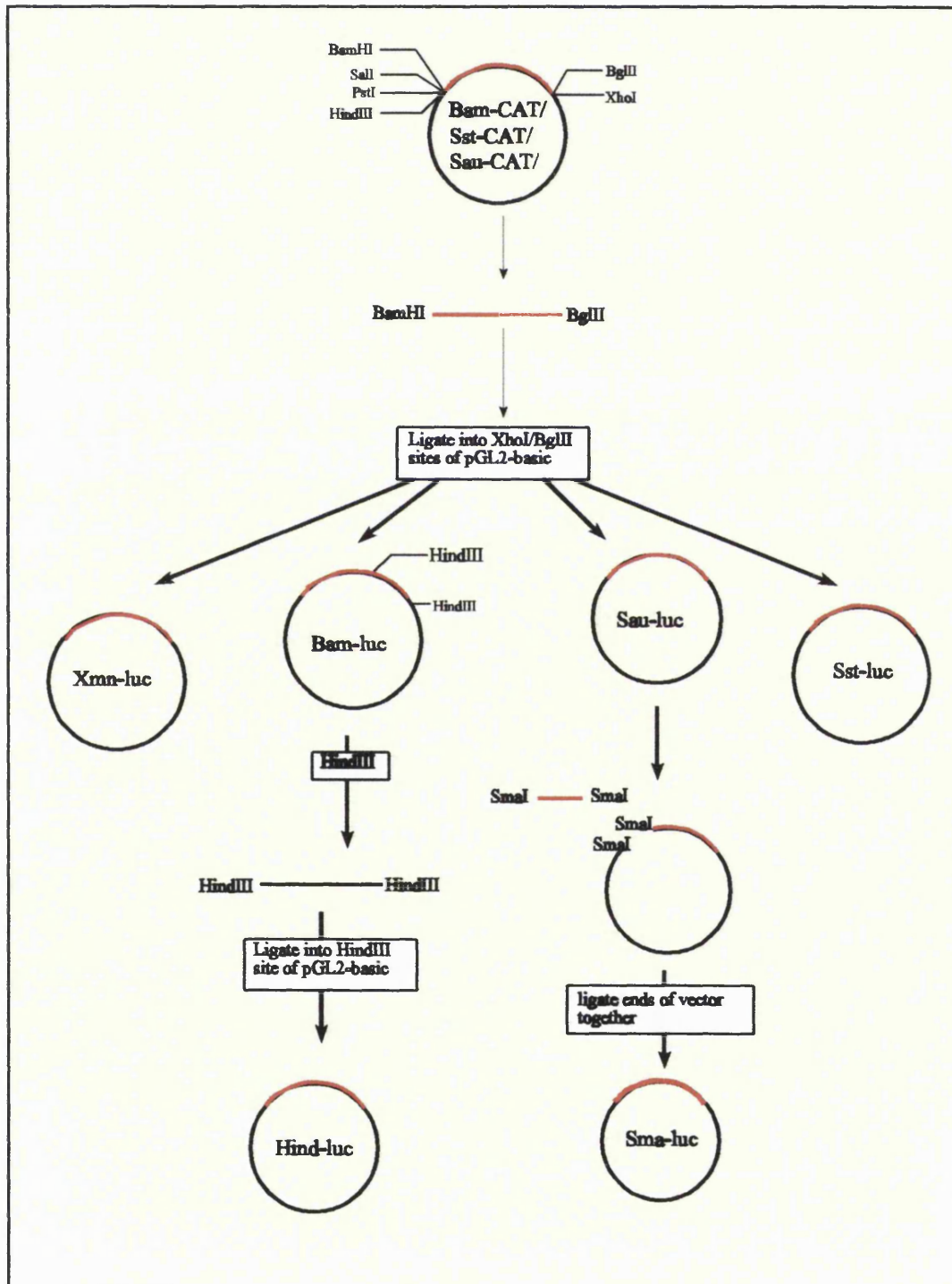
Fig. 3.4. Construction of SAA2 5' flanking region reporter constructs

and BglII to remove the fragment from the CATreporter vector, the fragment was cloned into the XhoI and BglII sites of the pGL2 basic vector. The Sma construct was made by digestion of the Sau-luc construct with Sma to remove the SAA2 5' flanking region 5' of the SmaI site. The ends of the construct from which the fragment was removed were religated back together.

3.2.1. Ligation reactions

Digested DNA fragments and vectors were isolated by electrophoresis through a 1% agarose gel, excision of the required DNA band from the gel and subsequent removal from the agarose using the Qiaex extraction system from Qiagen. Ligations were performed in a reaction mix containing DNA as specified below, 1x ligase buffer (supplied with enzyme) and 1 unit T4 DNA ligase. The reaction mix included 50 ng of vector and approximately 150ng of insert , to give a insert to vector molar ratio of approximately 5:1. To religate the ends of constructs of which regions are to removed the reactions contained approximately 50ng of the construct to be religated. The ligation reactions, at a total volume of 10μl, were carried out at 14°C for approximately 16 hours.

3.2.2. Transformation of bacteria

The ligated DNA's were transformed into 'One Shot' competent cells bacterial strain INVαF, considered to be equivalent to the strain DH5αfrom invitrogen. β-mercaptoethanol was added to the cells to a total concentration of 0.02M and incubated on ice for 2 mins. To a 20μl aliquot of the cells was added 5μl of ligation reaction mix, followed by incubation on ice for 30 mins. The cells were heat shocked at 42°C for 45 seconds and then incubated on ice for a further 2 mins. To the cells was added 400μl of SOC media (see section 2.4.1.) and the cells were incubated at 37°C for 1 hour with shaking at 225rpm. 200μl of the cells were plated onto LB plates containing ampicillin and

the plates incubated at 37°C overnight.

3.2.3. Colony PCR

Bacterial colonies from transformation plates were screened for positive clones by colony PCR. Colonies were picked using a inoculating needle. The needle was then 'dipped' into a 0.5 ml eppendorf tube containing a 1 x PCR buffer (see section 2.3.), 250ng pGL primer 1, 250ng pGL primer 2 (these oligos flank the pGL2 polylinker region fig 3.2) and 1 unit of Taq polymerase in a total reaction volume of 50μl. The remaining cells on the inoculating needle were used to inoculated 1ml of LB (see section 2.4.1.) with ampicillin and incubated at 37°C with shaking at 225 rpm. A PCR reaction is also carried out under the same conditions using the pGL2-basic vector only as a control reaction. PCR reaction conditions were as follows:-

95°C for 1 min

65°C for 1 min

72°C for 1 min --- for 30 cycles ↑

72°C for 10 mins

Positive clones were identified by agarose gel electrophoresis . An aliquot of each of the PCR reactions, and the control 'vector only' reaction was run an a 1% agarose gel. Positive clones were identified by a PCR product of a higher molecular weight than that for the vector alone.

3.2.4. Small scale preparations of DNA

Cultures from clones shown to be positive by colony PCR were used to make small scale plasmid DNA preparations (Brindoim and Doly, 1979). 100μl of the 1ml culture grown

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for colony PCR was used to inoculate 10mls LB containing ampicillin and incubated at 37°C, with shaking at 225rpm, overnight (approx 16 hours). 3mls of the overnight culture was spun down and used to make plasmid DNA using Qiagen Qiaspin miniprep kits. The plasmid DNA was digested with appropriate restriction enzymes to confirm the clones were positive for the desired insert. Remaining culture from positive clones was used to make glycerol stocks of the constructs for use in making large scale DNA preparations of the constructs.

3.2.5. Sequencing of Clones

Construct inserts were confirmed by sequencing using the thermal cycle sequencing kit from New England Biolabs and the pGL1 and pGL2 primers form Promega the sites for which are located at each side of the pGL2 polylinker. The method is based upon the dideoxynucleotide chain termination method of Sanger et al, 1977.

3.2.6. Large scale preparation of DNA

Bacteria from glycerol stocks of reporter constructs was streaked on to LB plates with ampicillin and incubated at 37°C overnight. Single colonies were picked from the plates and used to inoculate 10 mls LB containing ampicillin. The cultures were incubated for approximately 4 hours at 37°C until the OD of the culture was between 0.4 and 0.5units (A_{600}), subsequently 5 mls of the culture was added to 100 mls LB containing ampicillin and incubated at 37°C for a further 1-2 hours. 50 mls of the culture was added to 500 mls terrific broth (see section 2.5.) with ampicillin, and the cultures incubated at 37°C with shaking at 225 rpm, overnight.

The 500mls of overnight culture was centrifuged at 8000xg for 15 mins in a GS3 rotor and sorvall. The bacterial pellet was resuspended in 20 mls of cold soln I (see section

2.4.3.). A pinch (approximately 30mg) of lysozyme was added to the resuspended bacteria and incubated at room temp for 15 mins. Following addition of 60 mls of solution II (section 2.4.3) the bacteria were mixed and incubated for 10mins on ice, 45 mls of cold solution III (section 2.5.3) was added and the mixture inverted to mix and incubated on ice for a further 30 mins. Bacterial cell debris was pelleted by centrifugation at 8000xg for 10 mins. The supernatant was removed and filtered through a muslin cloth to ensure debris was not transferred. DNA was precipitated with 0.6 volumes of isopropanol and incubation on ice for 30 mins followed by centrifugation at 15000xg for 15mins in a GSA superlite 1500 rotor. The DNA pellet was air dried for 30 mins, 11 mls of T.E pH 8.0 was added and the pellet left to redissolve for 30 mins at room temp. The plasmid DNA was purified and separated from nicked DNA and RNA which may be present using CsCl gradient. To the redissolved DNA was added 12g CsCl to give a concentration of 1.09 g/ml. Once dissolved 200μl of ethidium bromide solution was added. The samples were incubated at room temp in the dark for 30 mins and then transferred to Beckman Quick Seal 16 x 76 mm centrifuge tubes which were heat sealed and centrifuged in a 70.1Ti rotor at 270,000xg for 16 hours. The DNA band was removed with a needle and syringe and the CsCl gradient centrifugation repeated. The ethidium bromide was removed from the DNA prep with approximately 4 washes with butanol. The DNA was precipitated with isopropanol, centrifuged to pellet the DNA and once dried the pellet was redissolved in 1 ml T.E. Concentration of DNA was measured using a Hitachi U-1100 spectrophotometer.

3.3 Transient transfection of reporter constructs

Actively dividing cells were seeded in the wells of six well plates at 1-2 x 10^5, 3-4 days prior to transfection. Reporter constructs with varying length fragments of SAA (fig 3.5.) were transfected into cultured cells using the calcium phosphate precipitation method

(Graham and Van der Eb, 1973 and Wigler *et al* , 1977). A β-galactosidase reporter construct (RSV β-gal) was cotransfected with the luciferase reporter vectors to act as an internal control normalising for cell number and transfection efficiency. 10µg of luciferase reporter construct and 2µg of the internal control in a total volume of 175ul with TE (see section 2.6.) was mixed with 25µl of 2M CaCl in a 15ml tube. This mixture was then added slowly dropwise with constant swirling to 200µl 2x HBS at pH 7.12 (see section 2.5.) in a 15 ml polypropylene tube. The samples were left at room temperature for 30 mins to allow the calcium phosphate precipitates to form. The transfection mix was added dropwise to the seeded wells which had grown to approximately 80% confluency and contained 2mls of media.
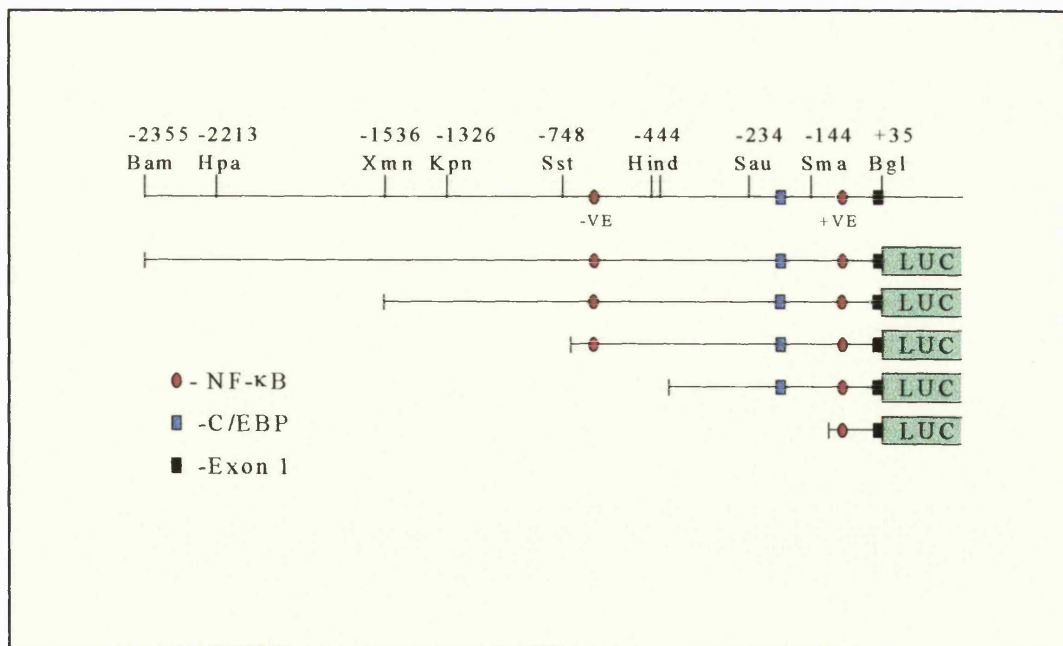
Fig 3.5. The SAA2 5' flanking regions used to make luciferase reporter constructs

The precipitate was left in contact with the cells for 16 hours . The cells were then washed twice with Hanks Balanced Salt solution and fresh media added to the cells. Cells were either left unstimulated or stimulated with the cytokines IL-1β and IL-6 at a concentration of 100U/ml for IL-1β and 1000U/ml for IL-6 for a period of 6 hours unless other wise stated.

3.3.1. Measurement of reporter construct activity

Cells were washed twice with Hanks Balanced Salt solution. They were then lysed by addition of 200μl of 1x Promega reporter lysis buffer to each well of the six well plates. The cells were incubated at room temperature for 15 mins before being put through one freeze thaw cycle to aid lysis and removal of cells from the plates. The cells were scraped from the wells using Sarstedt disposable cell scrapers and the lysate transferred to an eppendorf tube. The lysates were pelleted at 12,000 rpm for 5 mins to pellet the cellular debris, the supernatant was removed to a fresh tube and used to measure β-galactosidase and luciferase activity.

3.3.2. Measurement of β-galactosidase activity

β-galactosidase activity was assayed using the Galactolight Plus kit from Tropix. This assay system is similar to the systems used for measuring luciferase activity as it utilises a reaction which results in emission of light (Jain and Magrath, 1991). This reaction is catalyses by the enzyme β-galactosidase. The substrates for this reaction are trademarked and hence their compositions not documented within the product literature. To 20μl of cell lysate in luminometer tubes was added 200μl β-galactosidase reaction buffer. The samples were incubated at room temperature for 60 mins. 300 μl of accelerator II reagent

was added to the reaction and the light intensity of the reaction immediately measured on a bio-orbit model 1253 luminometer for a period of 5 seconds as recommended in the assay kit protocol.

3.3.3. Measurement of luciferase activity

Luciferase activity was measured using the luciferase assay kit from Promega. This kit is based on the reaction described in section 3.2. However the standard kinetics of this reaction result in production of light which occurs quickly and rapidly decays. The Promega system has been developed so that this light emission is constant for a longer period of time enabling easier measurement. The substrate used is beetle luciferin the reaction is carried out in the presence of Coenzyme A the result of which is that the intermediate formed is luciferyl-CoA as opposed to luciferyl-AMP (Wood, 1991). To 20µl of cell lysate in a luminometer tube was added 100µl of luciferase assay buffer (containing beetle luciferin and Coenzyme A, for full recipe see section 2.6.) and the light intensity of the reaction measured immediately as in section 3.3.2. for a period of 15 seconds. The light produced from the reaction is constant for a period of 20 seconds after which it begins to decrease, 15 seconds was chosen as the measurement time to give the maximum readings possible while remaining within this constant signal period.

3.4. Protein-DNA binding studies

3.4.1 Isolation and construction of DNA for protein binding studies

A 140bp fragment of DNA from -2213bp to -2355bp of the 5' flanking region of the SAA2 gene was isolated from the plasmid construct pKSHB2 . The construct, which was already available in the lab, is the pBluescript vector (fig 3.6) with the BamHI/HpaI

digested fragment (-2213bp to -2355bp) of SAA2 cloned into the BamHI/EcoRV sites.

The fragment was removed using the restriction enzymes BamHI and HindIII which both

leave 5' overhangs which can be radiolabelled in a reaction which fils in the 5' overhangs

with a radioisotope (section 3.4.2.)
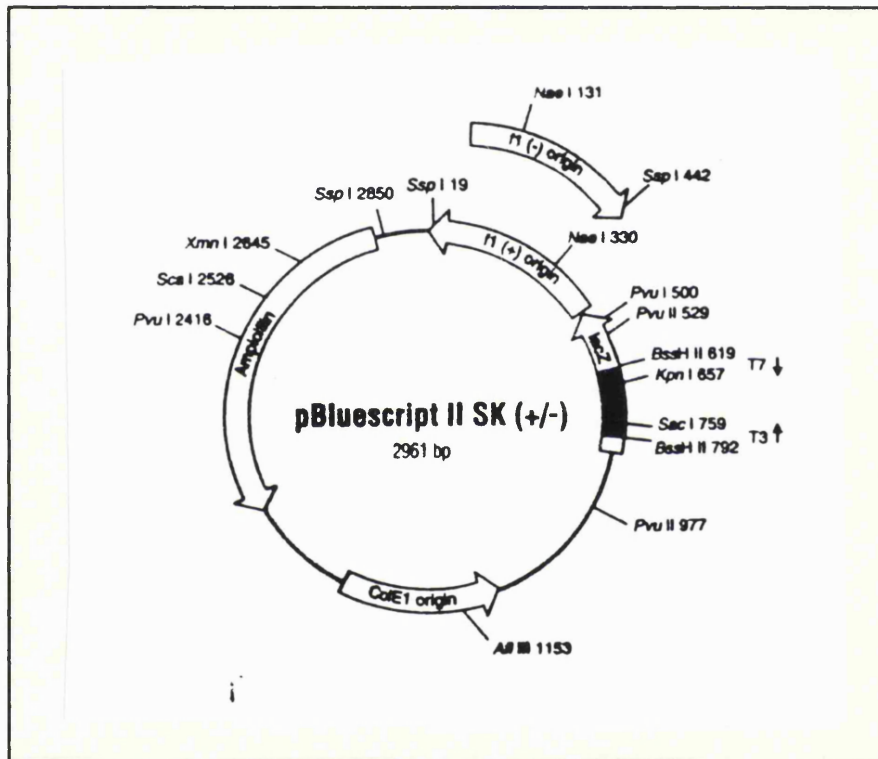
Fig. 3.6. The pBluescript vector

A 170bp fragment of DNA from -2313 bp to -2153 bp of the SAA2 5' flanking region was

generated by a PCR technique. The oligonucleotides pw270 and pw271 (fig 3.7) were

incubated with 10ng of the reporter vector Bam-luc, 1 x PCR buffer , 2mM dNTP's and

1 unit of Taq DNA polymerase.

The reaction conditions were as follows:

95°C for 40 seconds

72°C for 40 seconds

62°C for 40 seconds

for 35 cycles then,

72°C for 10 mins

The PCR reaction was run on a gel and the 170 bp band isolated using the Qiagen gel extraction kit

The isolated PCR products were cloned into the pBluescript vector using a TA cloning method. (fig 3.8). When Taq DNA polymerase is used in a PCR reaction an additional nucleotide is added onto the end of the copy strands resulting in a 1 nucleotide overhang in the resulting double stranded PCR product. This additional nucleotide is predominantly adenine. The pBluescript vector was modified to allow direct cloning of PCR products with adenine overhangs. pBluescript was digested with the restriction enzyme EcoRV which cuts the vector within the polylinker and leaves polished DNA ends. The digested vector was then incubated with Taq DNA polymerase, 1x PCR buffer and thymidine in order to add a single additional thymine to each end of the vector in order to create one nucleotide overhangs complementary to the PCR product overhangs. The reaction was carried out at 72°C for 10 mins. The vector was purified from unincorporated nucleotides by agarose gel electrophoresis and extraction using qiaex extraction kits. The PCR products were ligated into the modified pBluescript vector in a reaction which contained 1x ligase buffer, 1 unit T4 DNA ligase and vector and insert at a molar ratio of 10:1, the reaction was in a total volume of 10μl. The ligation was carried out at 14°C for 16 hours. A 5μl aliquot of the ligation reaction mixture was transformed using invitrogen one shot competent cells as described in section 3.2.2.

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3.4.2. Radiolabelling of DNA for protein binding studies

The Bam-Hpa fragment of the SAA2 5' flanking region and the 170 bp fragment generated by PCR with oligos pw270 and pw271 were radiolabelled in an 'end fill' reaction. The PCR product was cut from the Bluescript vector into which it was cloned using HindIII and BamHI restriction enzymes. The 5' overhangs of the DNA fragments were filled in with nucleotides in a reaction which contained 1x klenow buffer, 2 mM each of dGTP, dATP and TTP, 5mCi of α-dCTP P^{32}, and 1 unit of klenow. The reaction was incubated for 30 mins at room temp. The unincorporated nucleotides were remove using chromaspin -30 columns as described in section 3.1.1.

```
-2327
       GGGGACTCTATTCATCAGT  pw270
  TTATGGGGACTCTATTCATCAGTATTTACCATAATAGAAAGAAAA
  AATACCCCTGAGATAAGTAGTCATAAATGGTATTATCTTTCTTTT

  AATTTAAACATTTATCAATTCTTCAAAAAATTATCCATCTTCTAT
  TTAAATTTGTAAATAGTTAAGAAGTTTTTTAATAGGTAGAAGATA
    HpaI
  ATGTTAACATAAATAATATATTTTTATGAAAAATAACCATCTTCC
  TACAATTGTATTTATTTATATAAAAATACTTTTTATTGGTAGAAGG

  AAAAGAAAAAAAGTAAGAAGAGTACTATGGTTTTACCCTTTTT
  TTTTCTTTTTTTTCATTCTTCTCATGATACCAAAATGGGAAAAA
             pw271  CTCATGATACCAAAATGGGA
                                        -2149
```

Figure 3.7. Oligonucleotide sequence used to generate a fragment of SAA2 for use in protein binding assays
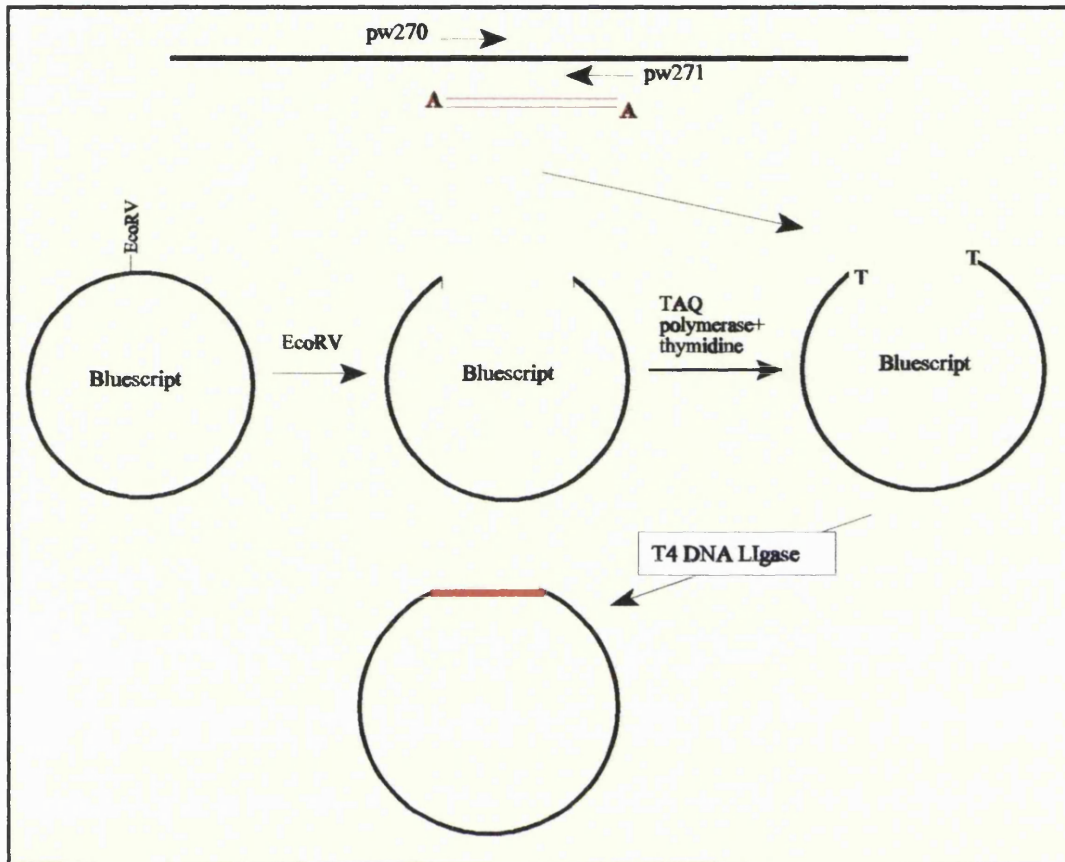
Figure 3.8. Construction of a pBluescript construct containing a region of SAA2 to be used in band shift analysis.

3.4.3. Isolation of nuclear extracts

Cells were seeded into 175cm^2 tissue culture flasks and were grown to 80% confluency (3-4 days). When confluent, serum containing media was removed and cells were grown in serum free media and either left unstimulated or stimulated with IL-1(100 units/ ml culture media) and/or IL-6 (1000 units/ml culture media). Cells were stimulated for time points ranging from 15 minutes to 24 hours prior to extraction of nuclear extracts.

Extracts were prepared based on a method by Dignam *et al*, 1983. To isolate nuclear extracts, cells were washed twice with cold (4°C) serum free media. The cells were then scraped from the flask with a cells scraper into the cold serum free media and removed to

a 50ml polypropelene tube. The cells were pelleted at 500xg for 5 mins at 4°C. All remaining stages were carried out at 4°C. After centrifugation the media was removed and the pellet suspended in 5 x the cell pellet volume of nuclear extract buffer A (section 2.9.) and the cells incubated on ice for 10 mins. The cells were then pelleted at 900xg for 5 mins at 4°C, the media removed and the cells resuspended in 3x the cell pellet volume of nuclear extract buffer A. Triton-X was added to the cell suspension to a final concentration of 0.05% to aid lysis of the cells. The cells were homogenised with a dounce homogeniser until approximately 90% lysis had been achieved as viewed under a microscope. The cell lysate centrifuged at 900xg for 10 mins at 4°C. The supernatant was removed to leave the nuclear pellet which was resuspended in an equal volume of nuclear extract buffer C. NaCl was then added the final concentration of 10mM or 300mM. The samples were incubated on ice for 30 mins with occasional gentle mixing. The extracts were then centrifuged at 1500xg for 30 mins. The supernatant containing the nuclear extracts was aliquoted into small volumes, flash frozen in liquid nitrogen and stored at -70°C.

3.4.4 Determination of protein content of nuclear extracts

The protein content of the nuclear extracts was determined by BCA assay. Bovine serum albumin was diluted to give a range of protein standards of known concentration to form a standard curve. The bovine serum albumin concentrations ranged from 0.1µg/ml to 1µg/ml. Samples were assayed in a 96 well plate, 10µl of sample at 1:10 and 1:5 dilution factors and 10µl of protein standard was assayed by addition of 200µl of BCA reagent containing 4% $CuSO_4$, and incubation of samples at 37°C for 30 mins. Samples were assayed at 562nm wavelength on an Anthos htII plate reader.

3.4.5. Electrophoretic mobility shift assays (EMSA)

The DNA was assayed for protein binding ability (Garner and Revzin, 1981)). DNA to be examined for protein binding properties was labelled as in section 3.4.2. The binding reaction contained approximately 20ng of radiolabelled DNA, 4µg of poly dI-dC (unless otherwise stated) to prevent non-specific binding of protein to the DNA, 1 x parker buffer and 10µg of nuclear extract. All components of the reaction except the radiolabelled DNA were incubated together for 10mins at room temperature prior to addition of the DNA. The samples were then incubated at room temperature for a further 20 mins before electrophoresis on a 5% non-denaturing polyacrylamide gel. Electrophoresis was carried out at 120V for approximately 3.5 hours, the gel was then transferred to 3MM whatman paper before being dried and autoradiographed at 70°C.

3.4.6. Electrophoretic mobility supershift assays

Supershift assays were carried out as for EMSAs with the exception that a rabbit polyclonal antibody to the transcription factor YY1 was added to the binding reaction either prior to addition of SAA2 DNA fragment or control oligonucleotide, or subsequent to addition of DNA. The binding reactions wee incubated with antibody for 20 minutes before progressing to the next stage. Control oligonucleotides were complementary DNA oligonucleotides corresponding to a YY1 consensus sequence.

3.5. Construction of SAA4 reporter construct

A SAA4 reporter construct containing approximately 100bp of human SAA4 5' flanking region and part of exon 1 was constructed using the pGL2 basic vector previously described. The SAA4 fragment was isolated from a larger clone by digestion with the restriction enzyme XbaI and subsequent ligation of the fragment into the NheI site of the pGL1 basic vector as this restriction enzyme produces 5' overhangs compatible with the

5' overhangs produced by the restriction enzyme XbaI. Cloning, screening and preparation of DNA was carried out as described for the construction of SAA2 reporter constructs. The SAA4 5' flanking region was sequenced using the Thermal Cycle sequencing kit from New England Biolabs.

4. Results

4.1 Analysis of SAA Expression in Multiple Human Tissues

Expression of all isoforms of serum amyloid A was believed until recently to be restricted to the liver, as is the case with the other acute phase proteins. However there have been a number of recent reports which have shown expression of SAA in both extra hepatic tissues and non-hepatic cell lines. In order to determine if the expression of SAA did exhibit tissue specificity the pattern of expression of both the acute phase and constitutive SAA isoforms were examined in a range of human tissues (figs 4.1.1 and 4.1.2). A multiple human tissue mRNA dot blot containing 50 different tissue samples and seven controls was probed with an oligonucleotide which hybridised to SAA1 and SAA2 (pw242) and an oligonucleotide specific for SAA4 (pw241). The autoradiograph results for both experiments are shown in figures 4.1.1b. (SAA1/SAA2) and 4.1.2b. (SAA4).

Visual analysis of the autoradiographs revealed a strong signal corresponding to the liver tissue sample with the SAA1 and SAA2 specific probe. However a signal was also detectable in all the other tissues tested albeit at a significantly lower level. No signals were observed for any of the negative control samples. Similarly with the SAA4 specific probe expression was detected for all the human tissue samples but the signal corresponding to the liver tissue sample is of a significantly higher intensity than for the other tissues. The difference in signal intensity between the liver and non-liver samples is not as great with the SAA4 specific probe as was observed for the SAA1/SAA2 specific probe.

(a)

	1	2	3	4	5	6	7	8
A	whole brain	amygdala	caudate nucleus	cerebellum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
B	occipital lobe	putamen	substatia nigra	temporal lobe	thalamus	subthalamic nucleus	spinal cord	
C	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
D	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
E	kidney	liver	small intestine	spleen	thymus	peripheral leucocyte	lymph node	bone marrow
F	appendix	lung	trachea	placenta				
G	foetal brain	foetal heart	foetal kidney	foetal liver	foetal spleen	foetal thymus	foetal lung	
H	yeast total RNA 100ng	yeast tRNA 100ng	E.coli rRNA 100ng	E.coli DNA 100ng	Poly r (A) 100ng	human C$_0$t1 DNA	human DNA 100ng	human DNA 500ng

(b)

Figure 4.1.1. Human multiple tissue mRNA dot blot (a) Human tissue samples and control samples present on the blot. (b) An oligonucleotide specific for human SAA1 and SAA2 was used to detect acute phase SAA expression.

(a)

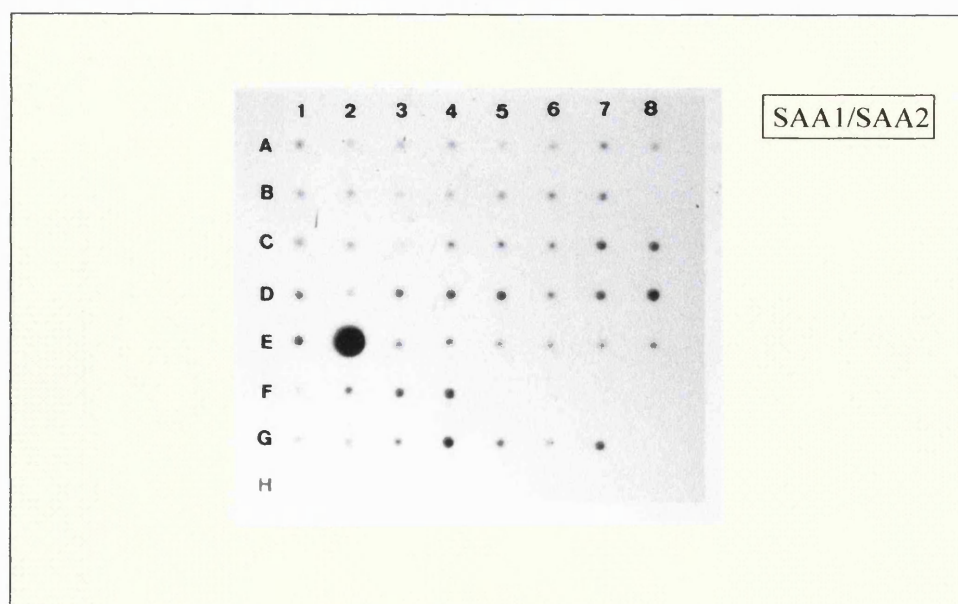|	1	2	3	4	5	6	7	8
A	whole brain	amygdala	caudate nucleus	cerebellum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
B	occipital lobe	putamen	substatia nigra	temporal lobe	thalamus	subthalamic nucleus	spinal cord	
C	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
D	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
E	kidney	liver	small intestine	spleen	thymus	peripheral leucocyte	lymph node	bone marrow
F	appendix	lung	trachea	placenta				
G	foetal brain	foetal heart	foetal kidney	foetal liver	foetal spleen	foetal thymus	foetal lung	
H	yeast total RNA	yeast tRNA	*E.coli* rRNA	*E.coli* DNA	Poly r (A) 100ng	human C_0t1 DNA	human DNA	human DNA

(b)
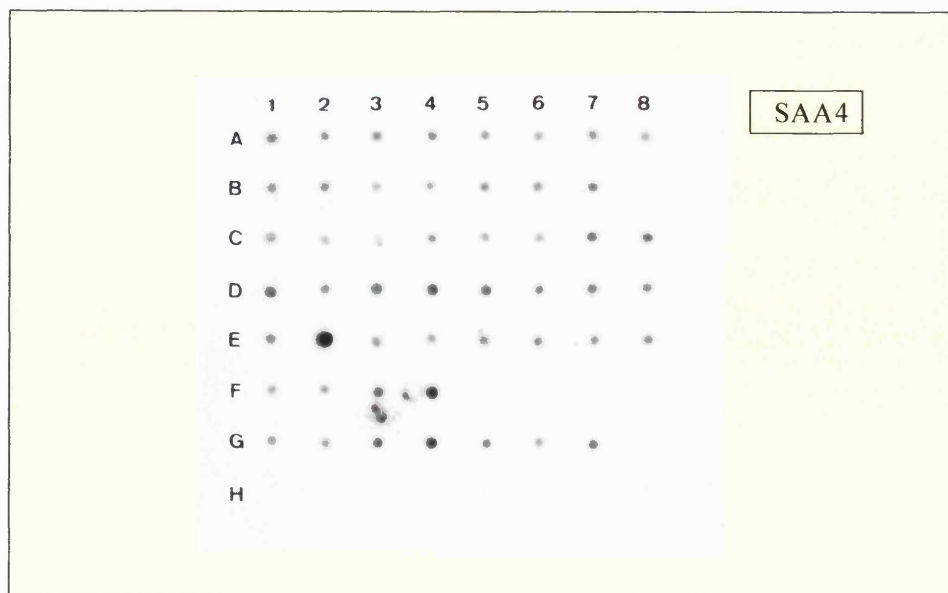
Figure 4.1.2. Human multiple tissue mRNA dot blot (a) Human tissue samples and control samples present on the blot. (b) An oligonucleotide specific for human SAA4 was used to detect expression of the gene.

The signal intensity was measured by densitometric analysis for each of the samples. The product information accompanying the blot addresses the issue of normalising the results obtained for RNA dot and northern blots. It is common to normalise RNA blots to the quantity of RNA in each sample by using equal amounts of each RNA however the manufacturers state that this does not take into account differences in transcriptional activity between different tissues. They state that under certain circumstances the proportion of housekeeping gene transcripts would be reduced in a given quantity of RNA due to the increase in the expression of particular genes depending upon the stimulus. In addition it was also noted that all housekeeping genes show some degree of tissue or developmental expression variations. To overcome these variations the manufactures of the dot blot have used eight different housekeeping genes to determine the quantity of RNA from each tissue to be loaded onto the blot so that a truly ubiquitously expressed gene with no tissue or developmental variation would be equally expressed in each sample on the blot. Accordingly, direct analysis of the densitometric results without subsequent normalisation to a single housekeeping gene would provide the most accurate results. The densitometric analysis of the SAA1/SAA2 expression levels are shown in figure 4.1.3. The level of expression in the liver tissue was significantly higher than for all the other tissue samples. All the other tissue samples exhibited low levels of SAA1/SAA2 expression.

Densitometric analysis of the SAA4 expression results (fig 4.1.4) revealed that like acute phase SAA (SAA1/2) the highest levels of expression were found in the liver, although the difference between the hepatic and non-hepatic expression was not as marked as for acute phase SAA. Also SAA 4 expression levels in the placenta were notably higher than the other non-hepatic tissues but significantly less than the hepatic expression.
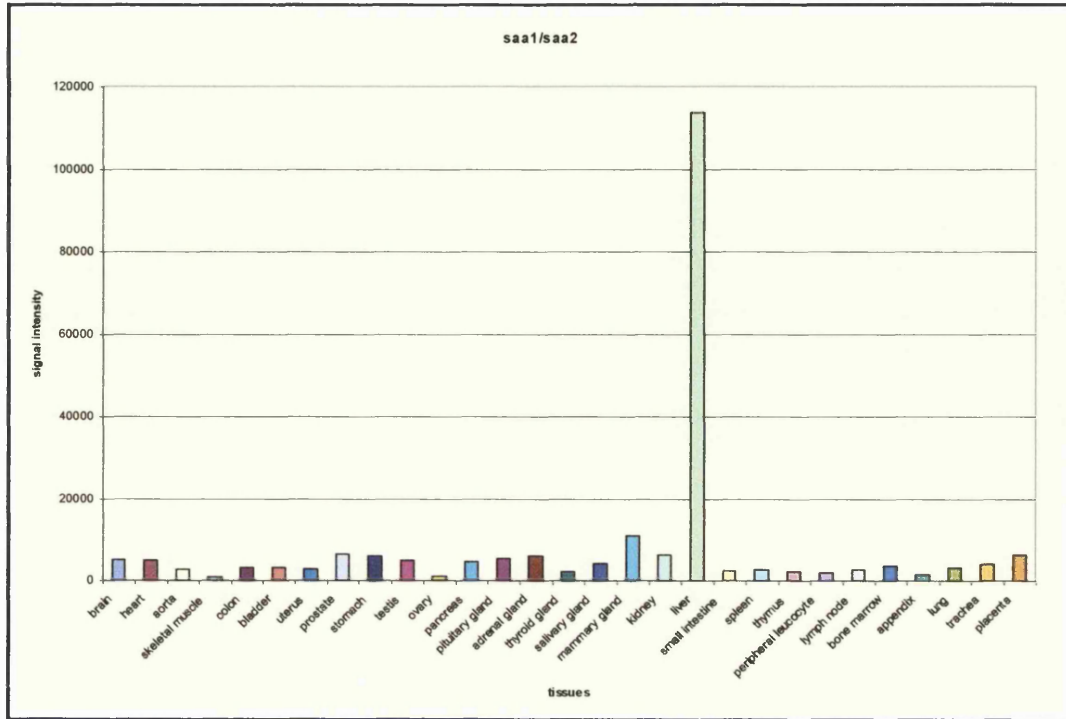
Figure 4.1.4. Densitometric analysis of the SAA1/SAA2 expression signals from the multiple human tissue mRNA dot blot.
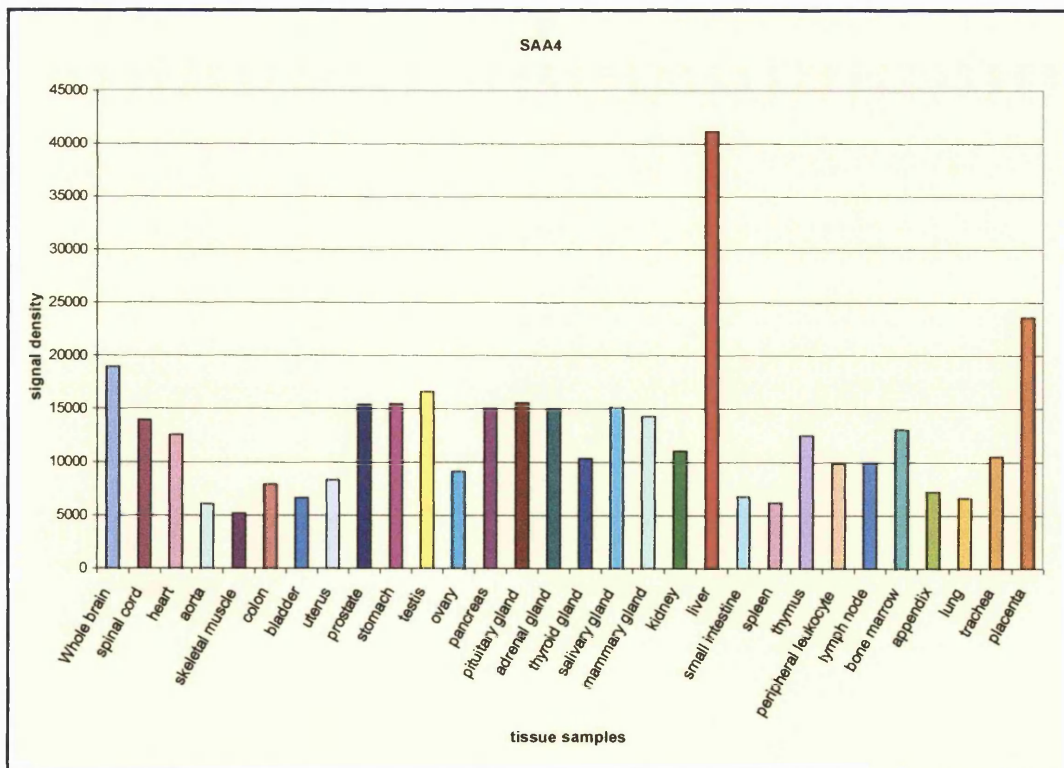
Figure 4.1.4. Densitometric analysis of the SAA4 expression signals from the multiple human tissue mRNA dot blot.

In addition to the adult/adolescent tissue samples present on the dot blot there were also a number of foetal tissues. The foetal tissues were also analysed by densitometric analysis to determine if the pattern of expression mimicked that of the
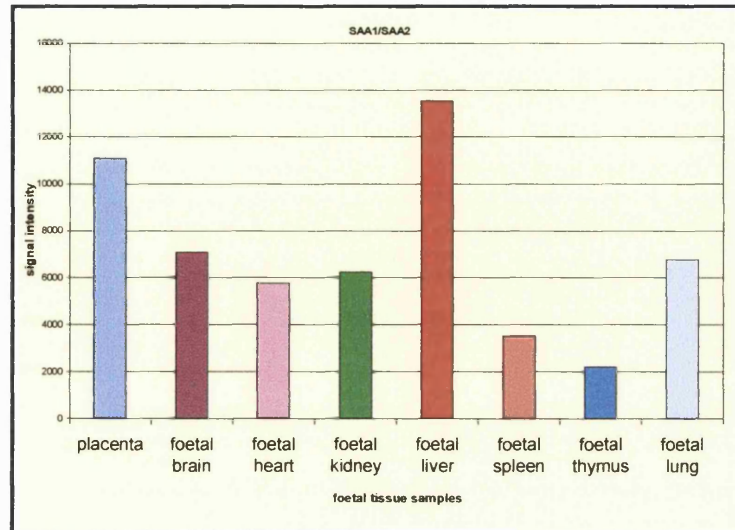
Figure 4.1.5. Densitometric analysis of SAA1/SAA2 expression in foetal tissues and placenta

adult/adolescent tissues (fig4.1.5). The SAA1/SAA2 tissue expression pattern did show the same profile as the non-foetal tissues in that the highest levels of expression were found in the liver and that all other tissues expressed SAA but at lower levels. Similar levels of expression were exhibited for the majority of the non-hepatic foetal tissues with the exception of foetal spleen and thymus for which the levels were marginally lower.

The SAA4 expression pattern in foetal tissues (fig 4.1.6) was also similar to the non-foetal tissues in that the highest levels of expression were found in the liver and the difference between this and the non-hepatic tissues was not as great as for the non-foetal
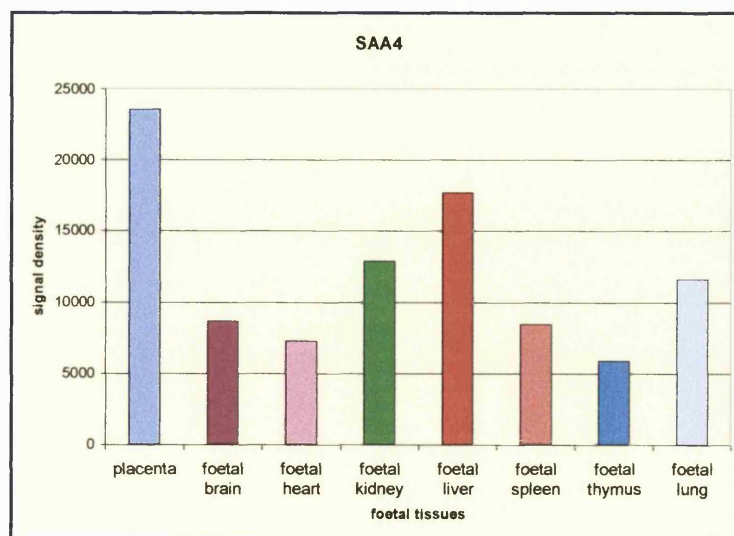
Figure 4.1.6. Densitometric analysis of SAA4 expression in foetal tissues and placenta.

tissues. The levels of expression of the different SAA isoforms in the placenta provide a

comparison for the foetal tissue expression levels to the levels found in the non-foetal tissues. The expression levels for all the foetal tissues fall within the range of expression for the non-hepatic adult/adolescent tissues.

Among the recent publications which have documented extra-hepatic expression of SAA was a report by Liang and colleagues (1997) who documented their findings of SAA expression in brain lesions of patients with Alzheimers Disease. Figure 4.1.7 shows the densitometric analysis of both the whole brain tissue the individual brain region tissue samples. Most of the various brain region tissue samples express similar levels of SAA1/SAA2 with the exception of the occipital lobe and putamen for which the levels are twice that of the majority of brain region tissue samples and cerebral cortex and frontal lobe for which expression levels were at least 50% less than the other brain region tissue samples. It is also interesting to note that SAA1/SAA2 expression was lower in all brain region tissue samples than in the whole brain - which would be expected to correlate most closely with the most abundant tissue of the brain.

Figure 4.1.8. shows the desitometric analysis of both the whole brain tissue regions and the individual brain tissue regions for SAA4. The pattern of SAA4 expression in the individual brain region tissue samples was very similar to SAA1/SAA2 with the exception of the occipital lobe and putamen. Although there were found to be variations in the levels of expression for the different samples there were no particular sample or samples that varied significantly from the others. As with SAA1 and SAA2 the individual brain region tissue samples exhibited lower levels of expression than the whole brain sample.

The nature of the tissue samples utilised for this dot blot means control of experimental conditions prior to isolation of RNA was not possible. The only information available about the source of the samples is cause of death which for each tissue donor was trauma.

No information is available regarding the status of the donors prior to death and it is therefore not known if these RNA samples represent the presence or absence of an acute phase condition. From the expression profiles it seems most likely that these results represent SAA expression during an acute phase response for two reasons. Firstly SAA1 and SAA2 expression is at barely detectable levels in the absence of an acute phase response whereas expression levels from the blot liver RNA sample were readily detectable. Secondly the extra hepatic expression has been reported in response to various stimuli and therefore the finding of extra hepatic expression of SAA suggests acute phase response conditions.
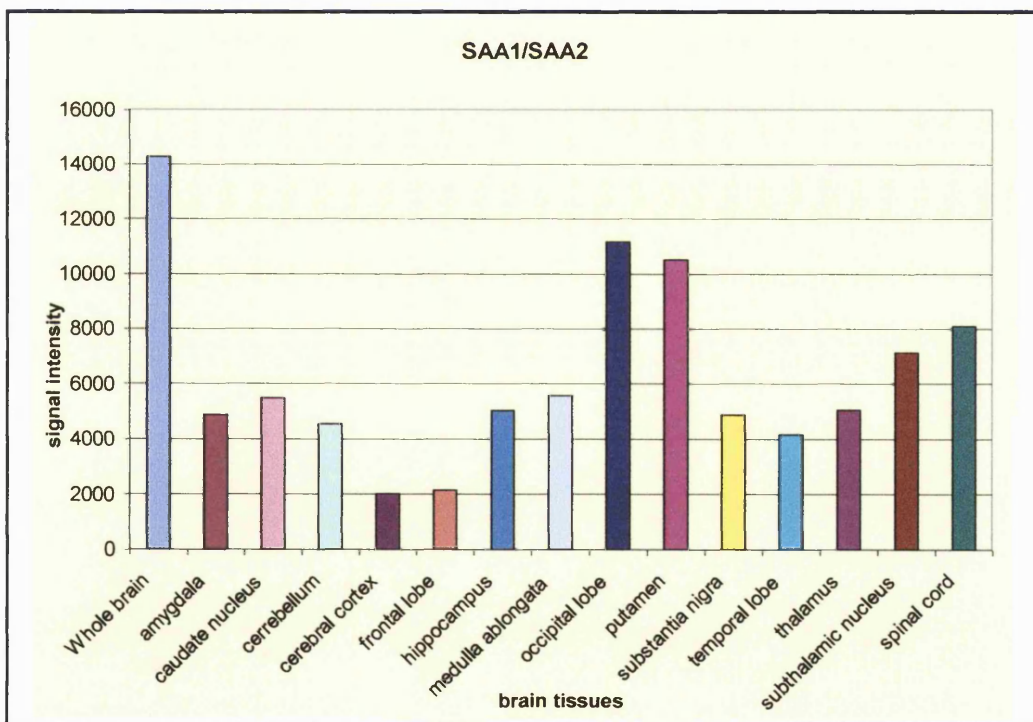
Figure 4.1.7. Densitometric analysis of SAA1/SAA2 expression in whole brain tissue and tissue samples from individual brain regions.

The dot blot results suggest that although SAA can be expressed extra hepatically thepredominant site of expression of both the acute phase isoforms and the constitutively expressed isoform is the liver. This indicates that SAA does exhibit tissue specificity and that mechanisms are present which enable this high level of hepatic expression while restricting expression in other tissues. Variations in the levels of extra-hepatic expression for some tissue samples may have implications in the pathogenesis of particular disease disorders in which extra hepatic expression of SAA has been proposed to be pathogenic.
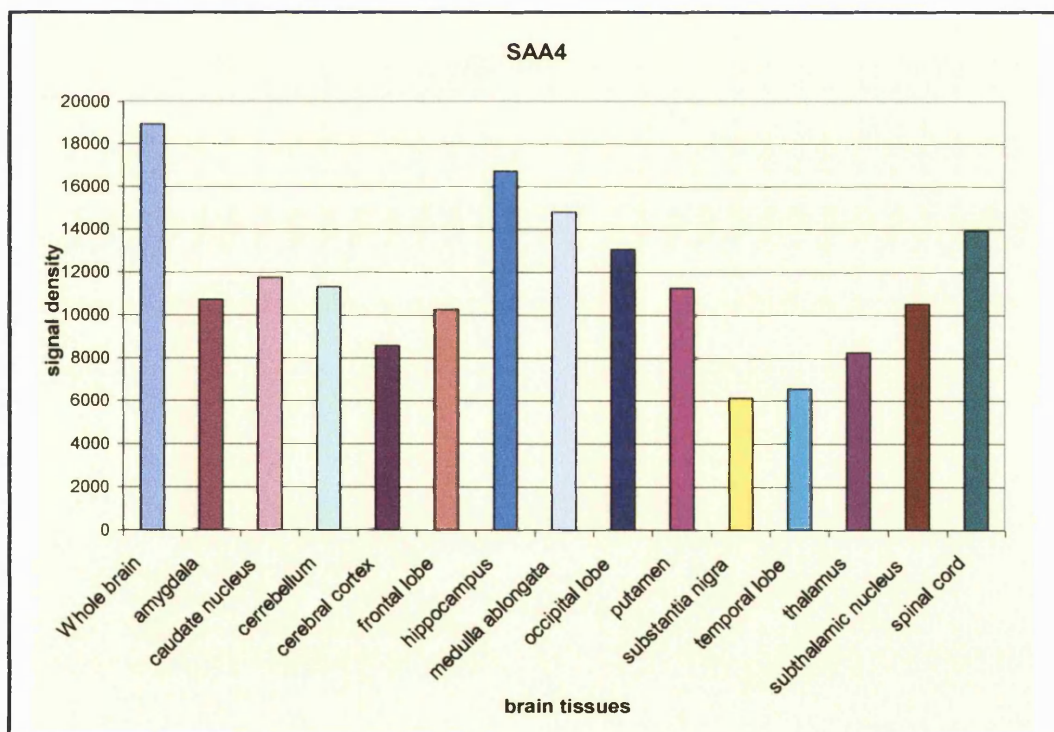
Figure 4.1.8. Densitometric analysis of SAA4 expression in whole brain tissue and tissue samples from individual brain regions.

4.2. Identification of regions of the SAA2 promoter containing tissue specific elements

4.2.1. Transient transfection assays with SAA2 reporter constructs

In order to identify the mechanisms responsible for the tissue specific expression of human SAA, transient transfection assays were carried out using luciferase reporter constructs containing varying length fragments of the SAA2 5' flanking region (fig 4.2.0). The SAA2 gene was chosen for these assays for the following reasons: SAA2 is the most extensively characterised gene of the human SAA gene family with regards to transcriptional control elements; there is also more sequence data available than for the human SAA1 and SAA4 genes. The human SAA1 and SAA2 genes are highly homologous and this homology extends to the conserved HindIII site at -444bp of the 5' flanking region (using SAA2 numbering). Transcriptional control elements identified within the SAA2 gene that are within this homologous region are also present within the SAA1 gene. Beyond -444bp this homology breaks down and an NF-κB binding site present in SAA2, 5' of the region of high homology is not found in SAA1. If the elements mediating the tissue specific expression of SAA2 are found to be within this region of high homology with SAA1 then it is likely that these same elements would be responsible for mediating the tissue specific expression of SAA1. This would eliminate the need for some of the individual analysis of the SAA1 gene.

Varying length fragments of the SAA2 5' flanking region were used to make luciferase reporter constructs by ligation into the polylinker of the pGL2-basic luciferase reporter vector (fig 4.2.0). These constructs were used in transient transfection assays utilising the hepatic cell line HepG2 and the non-hepatic cell line HeLa. These assays were carried out in order to identify differences in expression patterns of the two cell lines which may identify the elements responsible for restricting high levels of SAA expression to the liver. The fragments used to make the SAA2 reporter constructs were identical to those used in the construction of the SAA2-CAT reporter constructs, utilised previously within this group, that led to identification of the transcriptional control elements previously mentioned. One of the main differences between the use of CAT and luciferase reporter constructs is the half-life of their respective products. Chloramphenicol acetyl transferase has a half life of 50 hours whereas luciferase has a half life of just 3 hours. This significant difference in the stability of the reporter gene products has particular relevance when investigating SAA2 gene expression due to the acute phase nature of the gene.
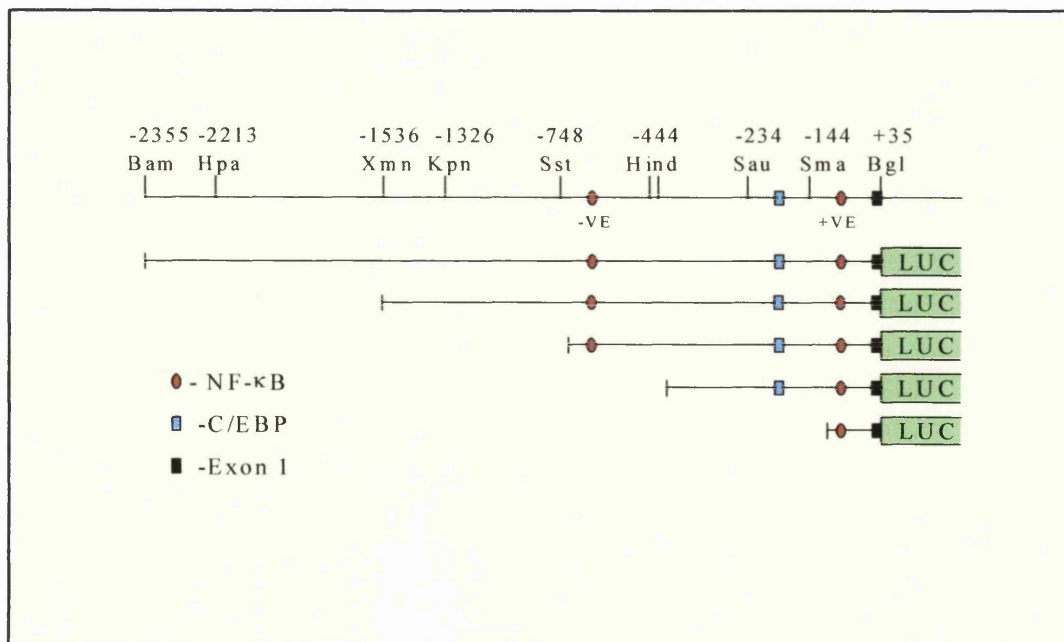
Fig 4.2.0. SAA2 reporter constructs used in transient transfection assays

In order to mimic an acute phase response *in vitro* the cytokines IL-1 and IL-6 were used to induce expression of the SAA2 reporter constructs. It is important when using cytokines or other agents to induce expression of reporter genes in transfection assays that the timing of cell harvest, which represents the time point at which reporter construct products will be assayed, coincides with the time at which maximal response to the stimulants would be observed. Due to the significant differences in the half-lives of luciferase and CAT, it was necessary to redefine the timing of the response to cytokines by the SAA2 5' flanking region in the luciferase vector.

A SAA2 luciferase reporter construct containing 35bp of coding region and 444bp of 5' flanking region was transiently transfected into the hepatic cell line HepG2 (fig 4.2.1.). Transfected cells were stimulated with IL-1β and IL-6 at concentrations of 100 and 1000 units per ml of culture media respectively. Cells were harvested at 0, 1, 3, 6, 10, 16 and 24 hrs after addition of cytokines. The results show a steady increase in the expression levels of the SAA2 reporter construct in response to IL-1β and IL-6 peaking at 6 hours followed by a steady decline. These results clearly show that the highest levels of luciferase accumulate at between 3 hours and 10 hours with the highest levels for this assay observed at the 6 hour time point. When SAA2 CAT reporter constructs were used in previous studies, cells were harvested at 16 hours after cytokine stimulation. These transfection assays show that at 16 hours the levels of luciferase produced from the SAA2 luciferase reporter construct Hind-luc which contains the C/EBP and proximal NF-κB binding sites would have peaked and would be returning to normal levels. As a result of these transfection assays it was decided to measure luciferase levels at 6 hours after stimulation with cytokines in subsequent transient transfection assays.
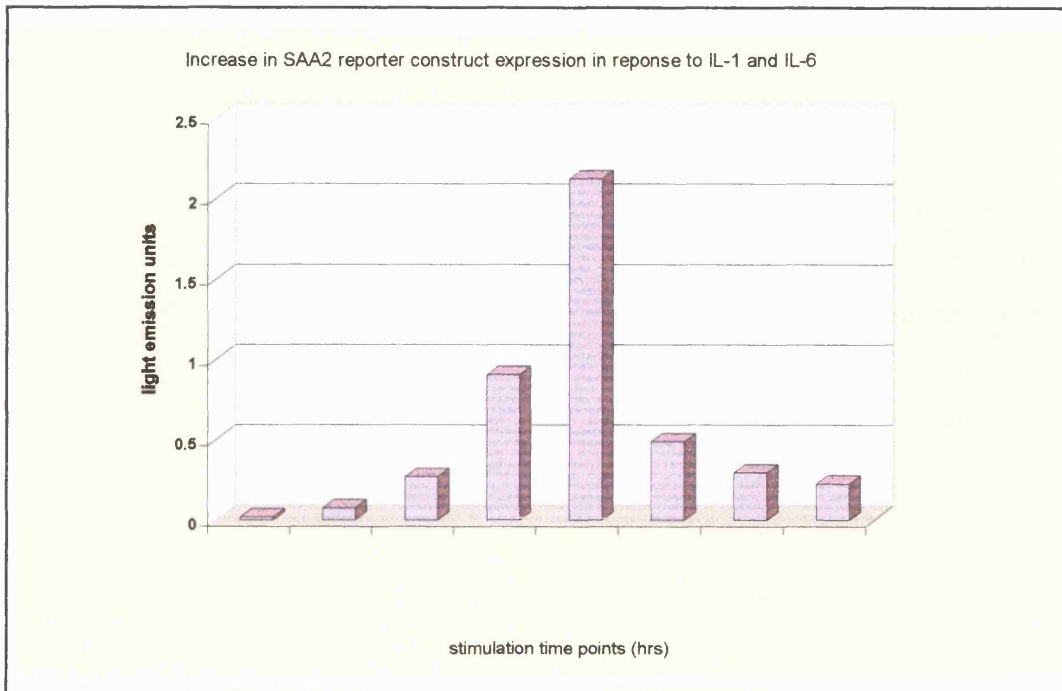
Figure 4.2.1. Time course results for the expression of the SAA2 reporter construct Hind-luc in response to IL-1 and IL-6

The SAA2 5' flanking region was analysed for the presence of tissue specific elements by transient transfection assays using reporter constructs containing fragments of the SAA2 gene extending to between -2355bp and -144bp of the 5' flanking region (see fig 4.2.0). As with previous transfections the constructs were transfected into HepG2 and HeLa cells left unstimulated or stimulated with IL-1β and IL-6. Figure 4.2.2. shows the basal and IL-1β and IL-6 induced expression of the SAA2 reporter constructs when transfected into HepG2 cells. Results are from three separate experiments each carried out in duplicate. Results were normalised for transfection efficiency and cell number by cotransfection with a β-gal reporter vector under the control of an RSV promoter. The results show all the SAA2 reporter constructs to be cytokine inducible. This is in accordance with the presence of a known cytokine responsive element, NFκB, 3' of the 144bp Sma restriction enzyme site. The results also show a drop in expression for reporter constructs containing the region from -444bp to -748bp, HindIII to SstI . This has been reported previously and is due to the presence of a negative regulatory element within this region. In addition the xmn

construct appears to show the presence of a positive regulatory region which appears to be surpressed by an element present by a negative element in the larger Bam construct .
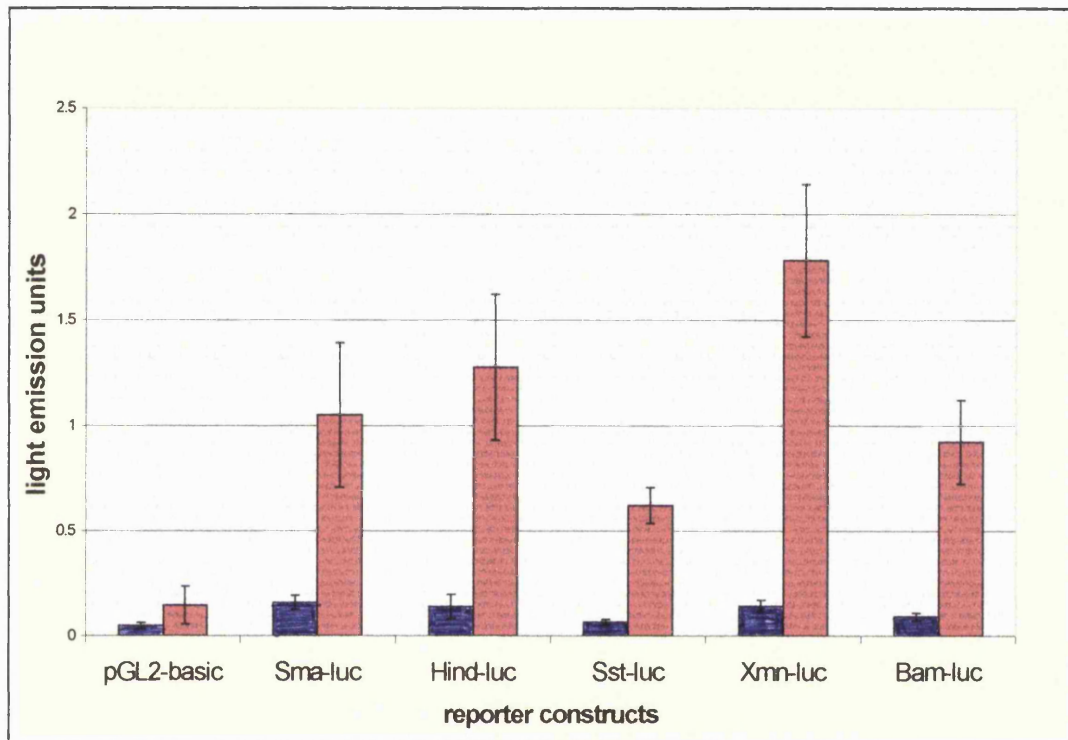
Figure 4.2.2. Expression of SAA2 luciferase reporter constructs transiently transfected into HepG2 cells. Cells were left unstimulated (blue bars) or stimulated with IL-1 and IL-6 (red bars)

Figure 4.2.3. shows the same reporter constructs transfected into HeLa cells. The results show that all the SAA2 reporter constructs are inducible in HeLa cells but to a lesser extent than in HepG2 cells. There is no decrease in expression levels in constructs containing the region from -444bp to -748bp, HindIII to SstI. Differences in expression of reporter constructs between the HepG2 and HeLa cell lines appear to be in the levels of induction in each cell line with the non-hepatic cell line showing a lower level of inducibility.

100
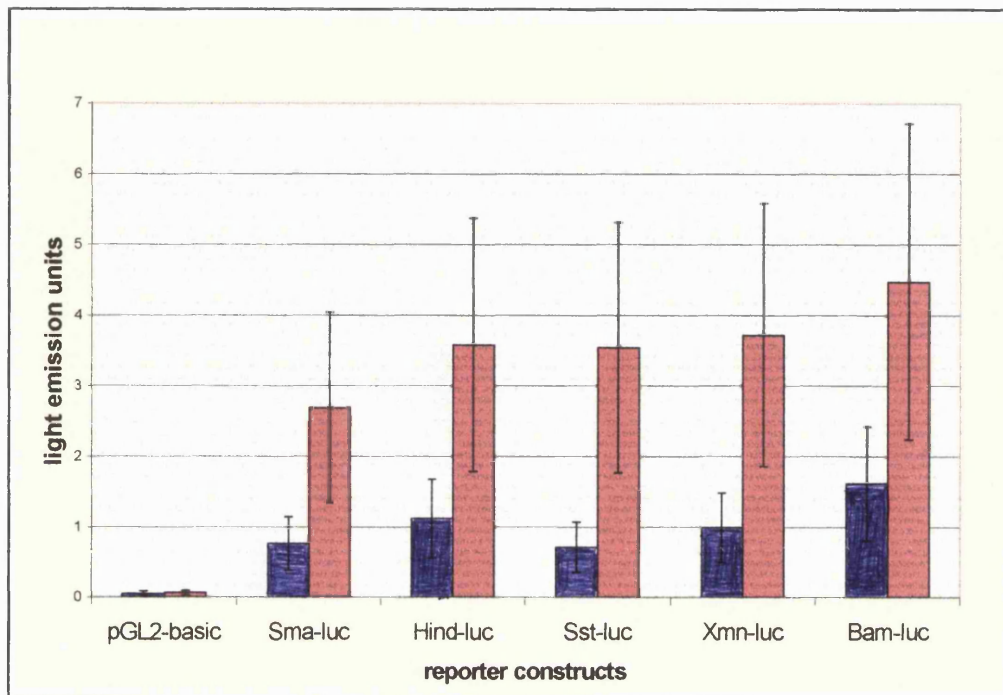
Figure 4.2.3. Expression of SAA2 luciferase reporter constructs transiently transfected into HeLa cells. Cells were left unstimulated (blue bars) or stimulated with IL-1 and IL-6 (red bars)

Direct comparison of the levels of reporter construct expression in each cell line is not possible. Different cell lines can behave differently and this includes differences in transfection efficiency. Normalising to a reporter vector such as a β-galactosidase expressing vector, while a widely used and useful method of normalisation, is not without it's problems. It has been found that constructs containing an RSV promoter are less efficiently expressed in HeLa cells than in other cell lines(Farr and Ronan, 1991). It has previously been found within this laboratory that constructs containing CMV promoters controlling the expression of a reporter genes such as β-gal have been found to be responsive to cytokines (unpublished data). Use of a normalising reporter construct containing a CMV promoter would therefore result in a distortion of the results when analysing cytokine inducibility. In order to try and overcome these difficulties and examine the results quantitatively, the expression levels for the two different cell lines were analysed

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as the level of induction of the reporter constructs. The mean values for basal and induced expression of the reporter constructs were used to determine the level of induction, known as the induction ratio, for each construct.

Figure 4.2.4. shows that the inducibility of the SAA2 reporter constructs in HepG2 cells varies from approximately 7 fold to 12 fold with the lowest ratio (7 fold) for the Sma-luc construct which contains the C/EBP binding site but no NF-κB binding site (all the other constructs contain both sites). There is no significant difference in the inducibility of the SAA2 reporter constructs containing both transcription factor binding sites in hepatic cells.
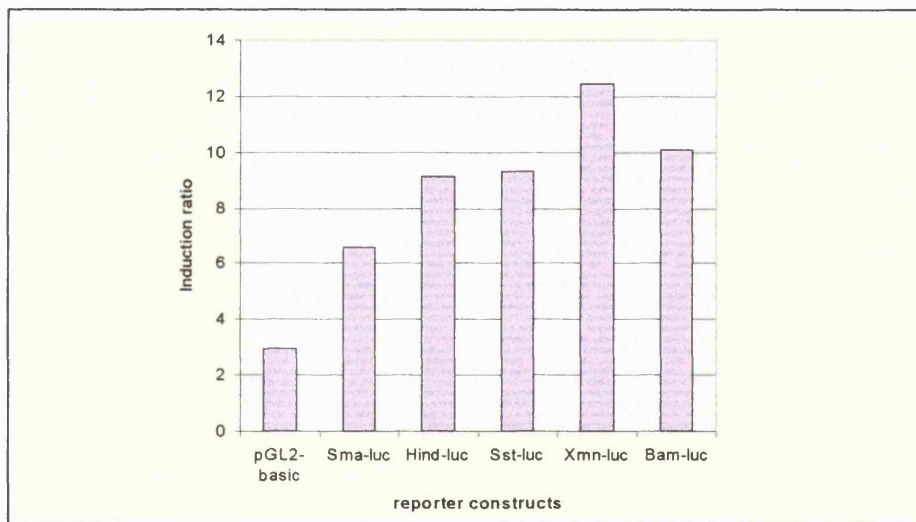
Figure 4.2.4. Induction ratios for SAA2 luciferase reporter constructs transiently transfected into HepG2 cells and induced with IL-1 and IL-6.

Figure 4.2.5. shows the induction ratio of these same constructs when transfected into the non-hepatic cell line HeLa. The induction ratios vary between approximately 2.7 fold and 5 fold however the lowest level of inducibility is observed for the Bam-luc construct which contains the largest SAA2 5' flanking region fragment extending to -2355bp. The level of inducibility of this construct is marginally lower then the Sma-luc construct, which was found to have the lowest inducibility ratio in hepatic cells. The presence of the region of the SAA2 gene containing the C/EBP binding site (SmaI to HindIII) does not cause the increase in inducibility found with HepG2 cells. C/EBP is one of the factors known as liver enriched

factors which have are known to be involved in liver specific expression. Additionally the transfection data also indicates that an element is present within the SAA2 5' flanking region which suppresses the inducibility of the gene in non-hepatic cells but not in hepatic cells.

These results confirm findings from preliminary studies carried out within this group by Janos Foldi. In these studies transient transfection assays were carried out using chloramphenicol acetyl transferase reporter constructs containing varying length regions of the SAA2 5' flanking regions (some of which were identical to the fragments used to make the luciferase reporter constructs). These constructs were transfected into HepG2 cells and human fibroblast cells. The results indicated that the IL-1 induced expression of the SAA2 reporter constructs was partially repressed in fibroblast cells when theregion of the gene from -2213bp to -2355bp was included in the reporter constructs.
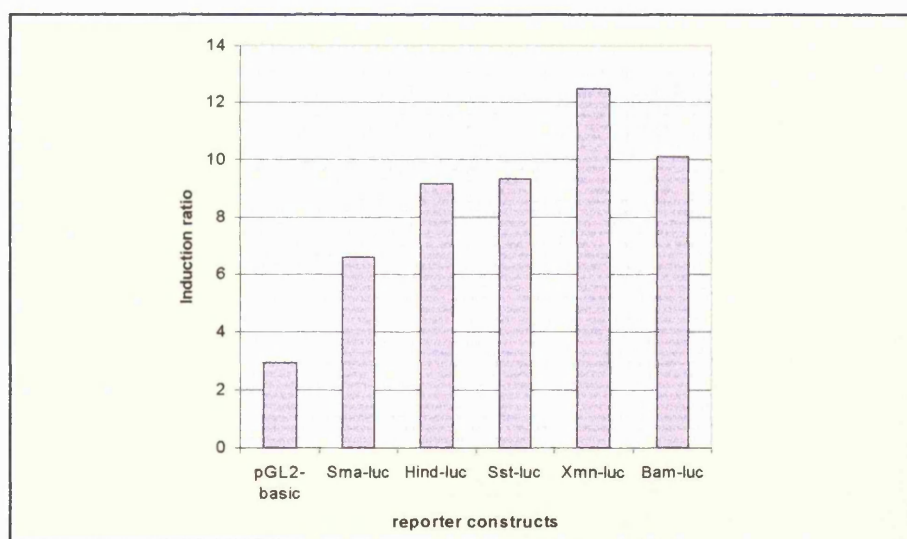
Figure 4.2.4. Induction ratios for SAA2 luciferase reporter constructs transiently transfected into HepG2 cells and induced with IL-1 and IL-6.

As a continuation from the results of the transfection studies detailed in this report and the work carried out by Dr Janos Foldi, the repressive capabilities of this fragment were investigated further by cloning this fragment into the pGL2-promoter luciferase reporter vector. This vector is identical to the pGL2-basic vector but also contains an SV40 promoter which drives transcription of the luciferase reporter gene. The Bam-Hpa SAA2 5' flanking region fragment (-2213bp to -2355bp) was cloned into the vector polylinker in both forward

and reverse orientations. The constructs were transfected as previously detailed and the results indicated by the expression induction ratio (fig 4.2.6.). In both the hepatic and non-hepatic cells the vector alone appears to show some inducibility of expression in response to IL-1 and IL-6. In the hepatic cell lines this inducibility is enhanced with the presence of the -2213bp to-2355bp fragment in an orientation dependent manner. In contrast in non-hepatic cells the presence of the fragment in the pGL2-promoter vector reduces the expression of the vector driven by the SV40 promoter in an orientation independent manner. These results confirm that the fragment of the SAA2 5' flanking region from -2213bp to -2355bp has tissue specific properties acting to suppress extra-hepatic expression of the SAA2 gene.
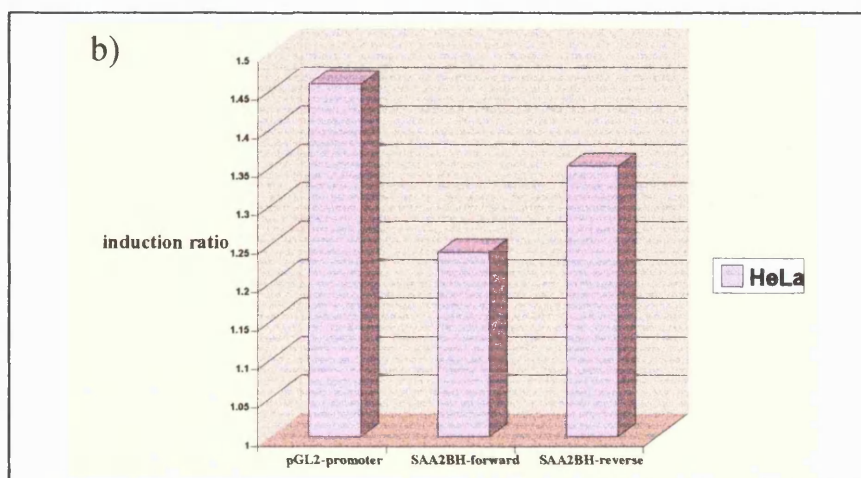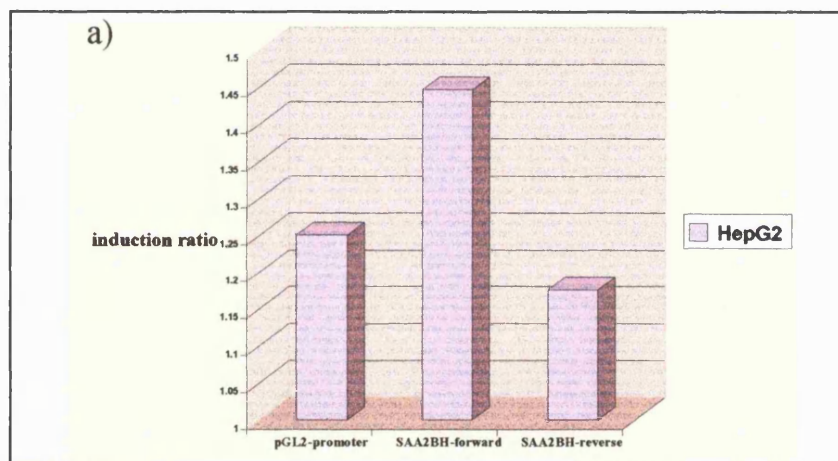
Figure 4.2.6. Expression of a SV40 promoter driven luciferase reporter construct containing a 140bp fragment of the SAA2 5' flanking region in the hepatic cell line HepG2(a) and the non-hepatic cell line HeLa (b)

4.3. Sequence analysis of a 140bp region of the SAA2 5' flanking region with tissue specific properties using the TFD and Transfac databases

The region of the SAA2 5' flanking region from -2213bp to -2355bp which had been identified as having tissue specific properties was analysed for possible transcription factor binding sites using the Transfac (fig 4.1) and TFD (fig 4.2) databases. The two databases differ in that while the Transfac database lists consensus sequences the TFD database lists specific published sequences and does not allow for degeneracy.

Factor or Site Name	Factor/site	Loc	Strand	Signal Sequence
silencer_bindin	site	5	(+)	TATTAAAA
TCF-1	fact	27	(+)	MAMAG
TCF-1	fact	34	(-)	MAMAG
vaccinia-term-s	site	52	(+)	CTATTC
gamma-IRE_CS	site	60	(+)	CWKKANNY
HiNF-A	fact	87	(+)	ATTTNNNNATTT
WAP_US6	site	88	(+)	TTTAAA
WAP_US6	site	88	(-)	TTTAAA
GMCSF_CS	site	94	(+)	CATTW
GATA-1	fact	97	(-)	WGATAR
CAP-site	site	101	(+)	CANYYY
Pit-1	fact	114	(+)	AWWTATNCAT
Pit-1	fact	116	(+)	WTATYCAT
CF1	fact	120	(-)	ANATGG
CAP-site	site	121	(+)	CANYY

Figure 4.1. Sequence analysis of the SAA2 5' flanking sequence using the transfac database

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Factor or Site Name	Factor/Site	Loc.	Strand	Signal Sequence
c-Myb	fact	16	(+)	ATTGAA
window_4	site	16	(-)	TCAAT
GR	fact	30	(-)	AGTTCT
c-Myb	fact	31	(+)	GAACTT
TBP	fact	36	(-)	ATAAA
unknown	site	36	(-)	AAATAAA
unknown	site	36	(-)	ATAAA
unknown	site	37	(-)	ATAAATAA
unknown	site	38	(+)	TATTT
Pit-1	fact	39	(-)	TAAAT
TBP	fact	40	(-)	ATAAA
unknown	site	40	(-)	ATAAA
AP-2	fact	44	(+)	TGGGGA
Sp1	fact	46	(-)	GAGTCCC
Pit-1	fact	53	(+)	TATTCAT
Pit-1	fact	53	(-)	ATGAATA
IUF-1	fact	57	(+)	CATCAG
unknown	site	63	(+)	TATTT
Pit-1	fact	64	(-)	TAAAT
Pit-1	fact	87	(-)	TAAAT
unknown	site	88	(+)	TTTAAA
unknown	site	88	(-)	TTTAAA
unknown	site	94	(+)	CATTT
unknown	site	94	(+)	CATTT
unknown	site	94	(+)	CATTT
Pit-1	fact	95	(-)	TAAAT
TBP	fact	96	(-)	ATAAA
unknown	site	96	(-)	ATAAA
GATA-1	fact	97	(+)	TTATCA
GATA-1	fact	97	(+)	TTATCA
GATA-1	fact	97	(-)	TGATAA
GATA-1	fact	97	(-)	TGATAA
factor_B2	fact	98	(+)	TATCA
GATA-1	fact	98	(-)	TGATA
NF-E1c	fact	98	(-)	TGATA
window_4	site	100	(+)	TCAAT
TFIID	fact	107	(+)	TTCAAA
GHF-1	fact	116	(+)	TTATCCAT
GR	fact	123	(+)	TCTTCT

Figure 4.2. Sequence analysis of the SAA2 5' flanking region using the TFD database.

Using the GCG program *Sigscan*, with each database, a list of possible binding sites which correspond to transcription factor binding sites reported in the literature were proposed. The search criteria specified a match for mammalian transcription factors only, in both cases. The searches each generated a list of transcription factors such as GATA-1 and sites such as CAP-sites.

The database searches provided some interesting results (fig 4.3). The Transfac database identified a CF1 consensus sequence at -2223bp to -2228bp. CF1 is alternatively known as YY1 a transcription factor which has been implicated in the expression of the rat SAA1 gene. It is displaced from the gene by the transcription factor NF-κB upon stimulation with IL-1. A YY1 site at this position is not identified from the transfac database nor are any other factor binding sites at this position. However, one particularly interesting result from this database search is the presence of a potential AP-2 binding site at position -2298bp to -2304bp. AP-2 is an IL-1 inducible transcription factor.

Figure 4.3. Sequence of the SAA2 5' flanking region from -2213bp to -2355bp and putative transcription factor binding sites identified using Transfac (red) and TFD (red) databases.

4.4. Examination of the protein binding properties of a 140bp region of the SAA2 5' flanking region

Although a number of consensus sequences for known transcription factors have been identified within the -2213 to -2355bp region of the SAA2 5' flanking region, presence of a transcription factor consensus sequence does not always dictate binding of the corresponding factors. In order to identify protein interactions with the DNA fragment in question electrophoretic mobility shift assays (EMSAs) were carried out. Nuclear extracts were prepared from HepG2 cells and HeLa cells. The cells had either been left unstimulated or stimulated with IL-1β and IL-6 at concentrations of 100 units and 1000 units per ml of culture media respectively. Times of cytokine stimulations are indicated individually for each result but varied from 15 mins to 16 hours. Nuclear proteins were isolated either in a high salt buffer (300nM NaCl) or a low salt buffer (10 nM NaCl) as indicated.
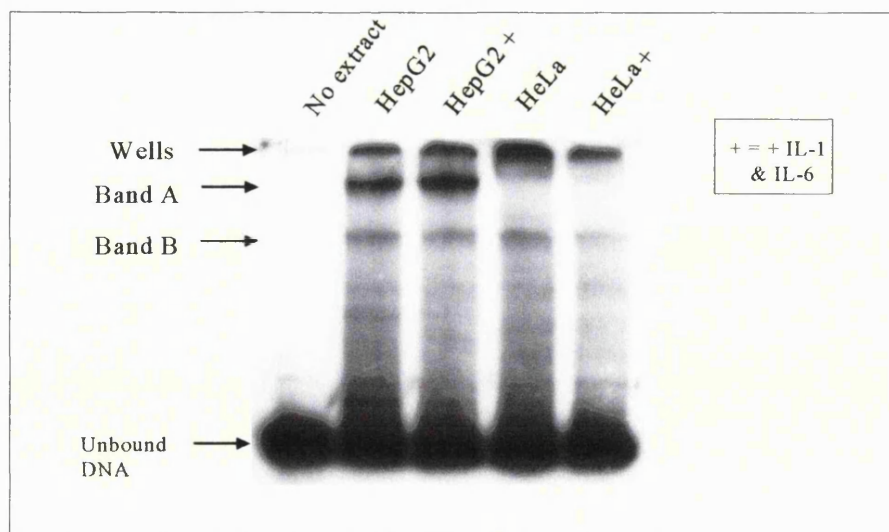
Figure 4.4.1. A 140bp fragment of the SAA2 5' flanking region was analysed for it's ability to bind protein present in nuclear extracts of HepG2 and HeLa cells which had either been left unstimulated or stimulated with the cytokines IL-1(100U/ml) and IL-6(1000U/ml). Nuclear extracts were isolated in a high salt concentration buffer.

The 140bp fragment of the SAA2 5' flanking region (-2213bp to -2355bp) which had been identified by transient transfection assays as having tissue specific properties was examined for its ability to bind proteins from nuclear extracts derived from the hepatic cell line HepG2 and the non-hepatic cell line HeLa. The fragment was radiolabelled as described in section 3.5.2 and then incubated with Nuclear extracts from both HeLa and HepG2 cells which had been left unstimulated and also from cells which had been stimulated with IL-1β and IL-6 for 16 hours (fig 4.4.1). The results show that this fragment does interact with proteins present in the extracts. A DNA-protein complex indicated by band B is present for each of the four extract samples, HepG2 and HeLa both stimulated and unstimulated. In addition a second complex band A is also present for the HepG2 samples both stimulated and unstimulated with IL-1 and IL-6. It appears from these results that the region of the SAA2 5' flanking region from -2213 to -2355 is able to bind a ubiquitously expressed protein in addition to binding a liver specific protein. Both these proteins appear to be constitutively expressed.
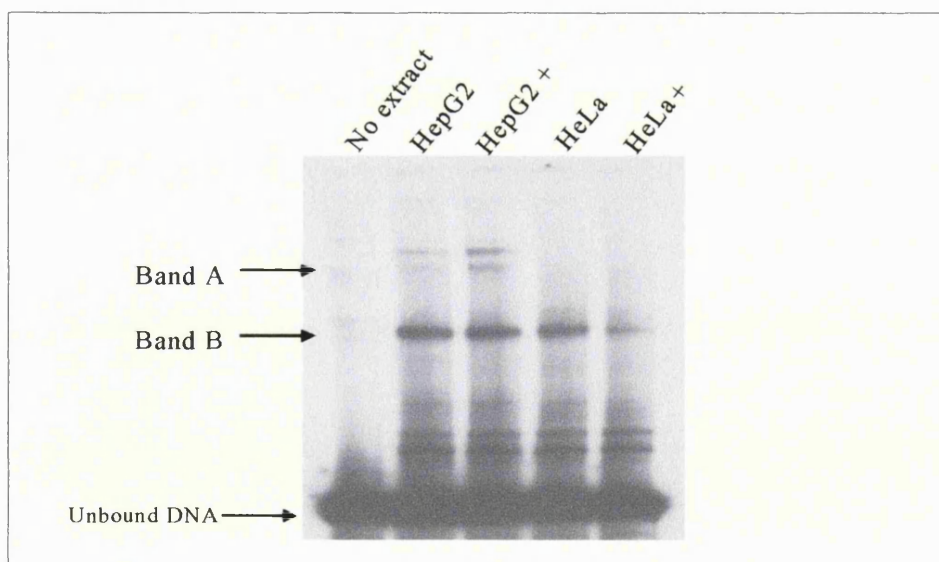
Figure 4.4.2. A 140bp frgament os the SAA2 5' flanking region was analysed for it's ability to bind protein present in nuclear extracts of HepG2 and HeLawhich had either been left unstimulated or stimulated with the cytokines IL-1(100U/ml) and IL-6 (1000U/ml). Nuclear extracts were isolated in a low salt concentration buffer.

One of the factors which can affect DNA protein interactions in DNA-protein binding assays is salt concentration. As the previous EMSA was carried out with proteins extracted in a high salt buffer the assay was repeated with protein isolated in a low salt buffer to determine if the DNA-protein complex formation differed (fig 4.4.2). This was primarly carried out to determine if further complexes could be detected which could not be formed with the high salt extracts. The EMSA showed the same banding pattern and hence DNA-protein complex formation as the EMSA for the high salt extracts, the DNA fragment binding to a ubiquitously expressed protein and also to what appears to be a liver specific protein.
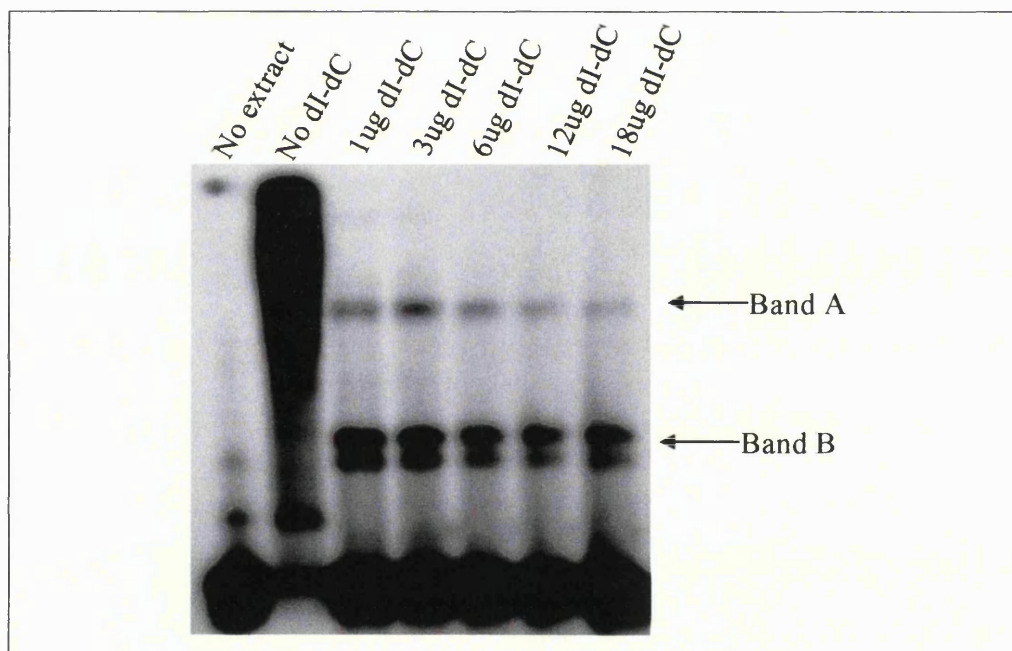
Figure 4.4.3. The effect of increasing concentration of poly dI-dC on the SAA2 fragment HepG2 protein complex formation.

In order to confirm that these bands represent specific binding two sets of competition experiments were carried out using polydI-dC and using cold oligonucleotide or DNA fragment competitor. Initially increasing quantities of poly dI-dC were used to try and

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compete non-specific binding of protein to DNA (fig 4.4.3). Poly dI-dC is a component of all the binding reactions however the quantity needed to prevent non-specific binding can vary between binding assays. Increasing the quantity of poly dI-dC without a visible change in the presence of DNA-protein complex bands indicates that these bands dorepresent specific binding and are not due to an insufficient quantity of poly dI-dC to completely prevent non-specific binding in that particular reaction. In the previous assays the quantity of dI-dC used in each binding reaction was $6\mu g$ for the competition experiments the quantities ranged from 0 to $18\mu g$. The binding assays were carried out with nuclear extracts from HeLa cells which had not been stimulated with cytokines.

When poly dI-dC is absent from the binding reactions very little unbound probe is visible but there is an absence of distinct protein-DNA complex bands indicating a large degree of non-specific binding. With addition of just $1\mu g$ of poly dI-dC there is an increase in the intensity of the band corresponding to unbound DNA and the appearance of the distinct DNA bands observed in the previous assays with the exception that the lower band appears as a double band this could be due to the presence of a degradation product of the protein which is involved in formation of this complex. Alternatively it could represent a third protein complex but this complex is not always observed. The presence of such double bands has been occasionally been observed for the different bands in different experiments and does not appear to represent a further protein-DNA complex. Although there do appear to be slight variations in the intensity of the bands between lanes the bands could not be competed out even with concentrations of poly dI-dC as high as $18\mu g$.

Competition experiments were also carried out using varying quantities of unlabelled DNA fragment in the binding reaction in addition to the set quantity of radiolabelled DNA probe (fig 4.4.4). Unlabelled DNA competitor was present in the binding reactions at concentration of 1, 10, 25 and 100 times that of the labelled DNA probe. The results show

a gradual decrease in the intensity of the DNA bands (both bands appear as a doublet in this figure) when the unlabelled competitor is present at a 100 fold excess the distinct specific bands are virtually absent. The two sets of competition experiments have shown that the bands observed in the EMSAs represent specific protein-DNA interaction and not non-specific binding of protein to DNA.
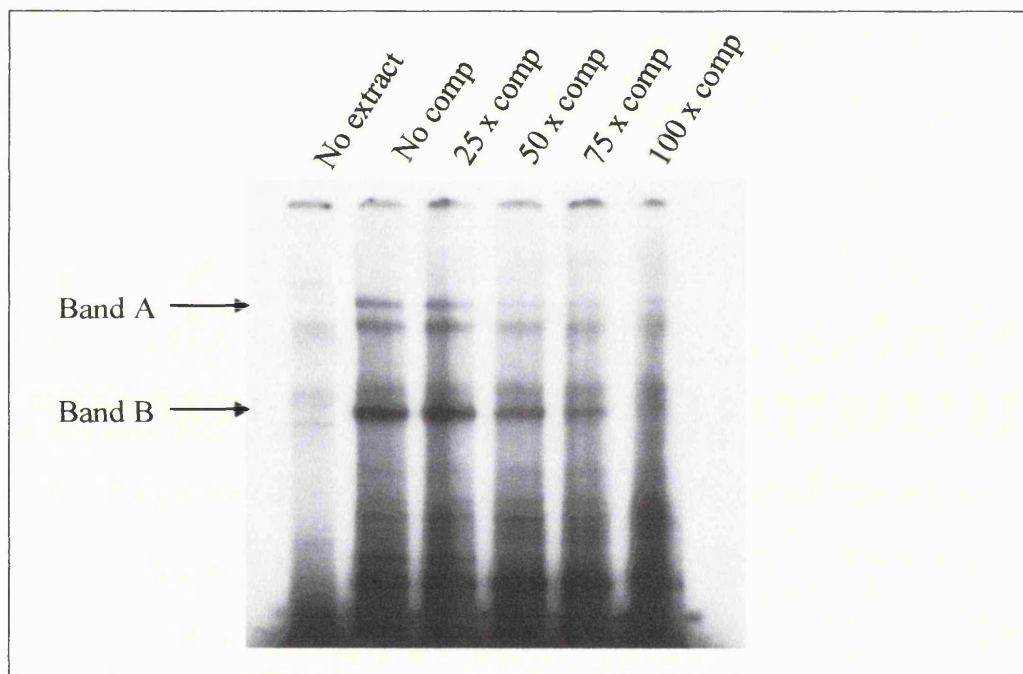
Figure 4.4.4. Abrogation of protein binding by competition with unlabelled SAA2 5' flanking region DNA.

Transient transfection assays have indicated that inclusion of the bam-hpa fragment in reporter constructs confers some degree of tissue specificity. It is possible that the regulatory element is present at the 3' end of the fragment effectively transversing the HpaI site which represent the 3' end of the fragment being utilised in the binding assays. If this was the case then it is possible that due to an incomplete DNA binding site at the 3' end of the fragment a protein which binds this region would be unable to bind and hence

would not be detected in the binding assays. In order to determine if further DNA binding

sites are present within this region a 170bp fragment was created by a PCR technique

which extended from -2313bp to -2153bp. This fragment was cloned into bluescript and

isolated by restriction enzyme digests for use in DNA-protein binding assays. When this

fragment was incubated with nuclear extracts from HepG2 and HeLa cells, which had

been left unstimulated or stimulated with cytokines, the results were found to be the same

as those observed when the -2213 to -2355 DNA fragment was used (4.4.5). This

indicates that protein binding is occuring within the DNA region shared by the two

fragments, i.e. the region of the SAA2 5' flanking region from -2213bp to -2313bp.
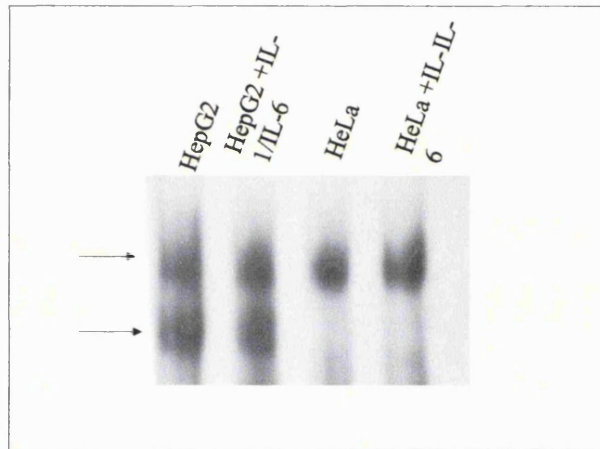
Figure 4.4.5. Electrophoretic Mobility Shift Assay
of SAA2 PCR Fragment transversing the -2215bp
HpaI site

Within this region (-2213bp to -2313bp) lies the consensus sequence for the transcription

factor CF1 which is now more commonly known as YY1. YY1 is known to be involved

in transcriptional regulation of the rat SAA1 gene. It is displaced from the SAA1 gene by

NF-κB upon stimulation with IL-1. In order to determine if this YY1 site was binding the

transcription factor YY1 electromobility shift assays were carried out using wild type

sequence oligonucleotides and mutated sequence oligonucleotides in order to determine

if mutation of the sequence would prevent formation of one of the DNA-protein

complexes previously observed. The consensus sequence as detected by the TFIID database is ANATGG where N represents any nucleotide. A consensus sequence for YY1 has been determined by analysis of a number of known YY1 binding sites. These consensus sequences exhibited some minor variations however there was one major factor which is common to all the consensus sequences and which appears by observation of the sequences to exhibit very little deviation or degeneracy and that is the presence of a core CCAT sequence within the binding site. This core sequence was used as the basis for the mutational studies.

Three sets of mutations were carried out. The substituted bases were chosen according to two criteria: firstly that, in so much as was possible, the substitute bases did not correspond to accepted degeneracy in the consensus sequences which had been reported; secondly that the resultant oligonucleotides had the same Tm. In addition to the mutant oligonucleotides, wild type sequence oligonucleotides were also created. The oligonucleotides (figure 4.4.6.) were annealed , radioactively labelled and used in EMSA's.

Wild type	CTTCAAAAAATTAT<u>CCATCT</u>TCTATGTT
Mutant 1	CTTCAAAAAATTATCCAT<u>TGA</u>CTATGTT
Mutant 2	CTTCAAAAAATTATC<u>TGA</u>CTTCTATGTT
Mutant 3	CTTCAAAAAATT<u>TAGC</u>ATCTTCTATGTT

4.4.6. Mutant and wild type sequence oligonucleotides used in EMSA assays

Mutant and wild type SAA2 5' flanking region oligonucleotides were incubated with nuclear extracts prepared from HepG2 cells and HeLa cells which had not been stimulated with cytokines (fig 4.4.7.). The results show a number of bands present for both the HepG2 samples and the HeLa samples, the number of bands exceeds that of the previous EMSAs using the 140 bp fragment as opposed to the oligonucleotide. This could be due to the presence of bands which represent non-specific binding or degradation products of a binding protein. The banding pattern that is observed is the same for both the HepG2 and the HeLa samples indicating that any transcription factor that binds within this region is not the liver specific factor that is found to bind the longer 140bp 5' flanking region fragment.

The banding pattern that is observed for the wild type sequence oligonucleotides is also observed for two of the sets of mutated oligos (mutants 1 and 2). However for the third set of mutated oligonucleotides there is a change in the banding pattern with the disappearance of complex C and the appearance of a complex D which migrates more quickly through the gel than complex C and hence represents a complex of a smaller size. There are two possible explanations for the appearance of this smaller sized complex. Firstly it is possible that the sequence of the mutant 3 oligo has coincidentally introduced a binding site for a different transcription factor of smaller sizethan that which forms complex C. Alternatively it is possible that complex C is composed of more than one transcription factor and as a result of mutation of the sequence prevents binding of one but not all of the protein components of this complex hence a DNA-protein complex is still formed but is smaller in size.
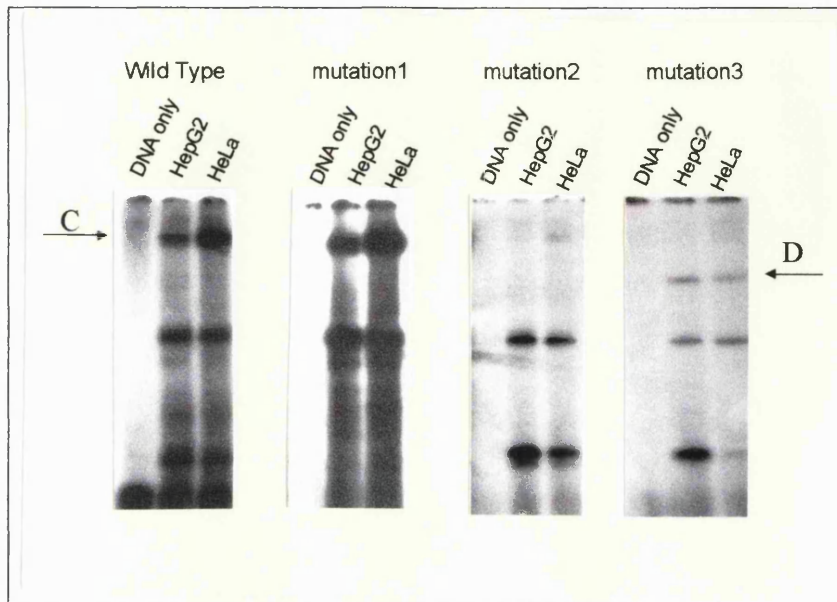
Figure 4.4.7. Analysis of the protein binding properties of a wild type and mutated YY1 consensus sequence present in the SAA2 5' flanking region.

Although these results show that mutation of the YY1 consensus sequence present in the 5' flanking region of the SAA2 gene effects the formation of DNA-protein complex C this does not prove that it is YY1 that is involved in formation of this complex. Confirmation of the identity of the protein component of this complex was sought using polyclonal antibodies to YY1. The antibodies were used to try to supershift the complex bands formed in the EMSA (fig 4.4.8). Incubation of the complex with the antibody should, if YY1 is a component, result in formation of a larger sized complex which will migrate more slowly on the gel due to binding of the antibody to YY1. Alternatively the antibody could bind to YY1 preventing its incorporation into the complex resulting in formation of a smaller band as found in the mutational studies. Figure 4.4.8. shows supershift assays carried out with a set of YY1 consensus oligonucleotides provided by Santa Cruz Inc. These oligonucleotides are not derived from the SAA2 sequence but conform to the YY1

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consensus sequence This supershift assay was carried out to confirm that the antibodies would bind to the factor YY1 under the conditions being utilised. The oligonucleotides were incubated with nuclear extracts prepared from HepG2 cells which had not been stimulated with cytokines. The antibodies were added to the binding reaction either prior to addition of oligonucleotide or after the addition of oligonucleotide to the binding reaction. The results show that when the YY1 antibody is added to the binding reaction prior to addition of the DNA, the band A disappears indicating than the antibody is preventing the YY1 binding to the DNA. When the antibody is added after addition of the DNA to the binding reaction there is no change in the banding pattern from that of the sample free of antibody added. This suggests the antibody is unable to bind sufficiently to cause a significant change in the size of the complex possibly because the antibody epitopes are located within the DNA binding region.
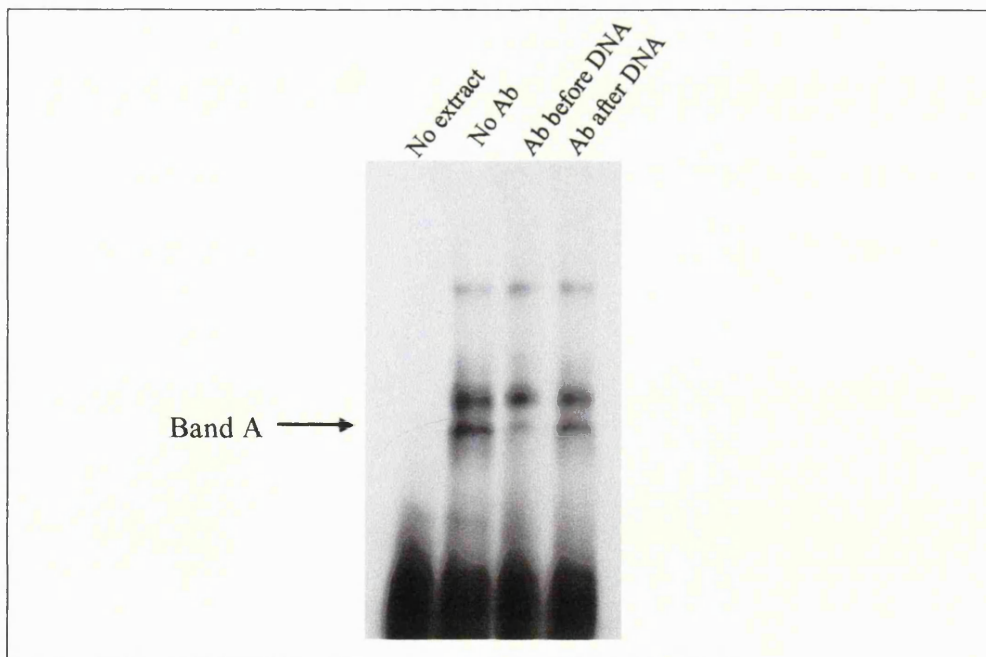
Figure 4.4.8. Supershift assay using YY1 consensus sequence oligos with known binding properties and polyclonal antibodies to YY1. Band A represent the specific YY1-DNA complex.

This electrophoreticmobility supershift assay was repeated using the 140bp SAA2 5'
flanking region DNA fragment (fig 4.4.9) The DNA-protein complex bands were not as
distinct as for previous assays and as a result of which the results are inconclusive. It
appears likely from the supershift assays with the YY1 consensus oligonucleotides that
addition of antibodies to YY1 would be more likely to prevent binding of YY1 to the
complex (if YY1 is in fact a component of the complex) and produce a change in the
banding pattern as observed for the mutational studies in that the band appears lower
rather than migrating more slowly through the gel.
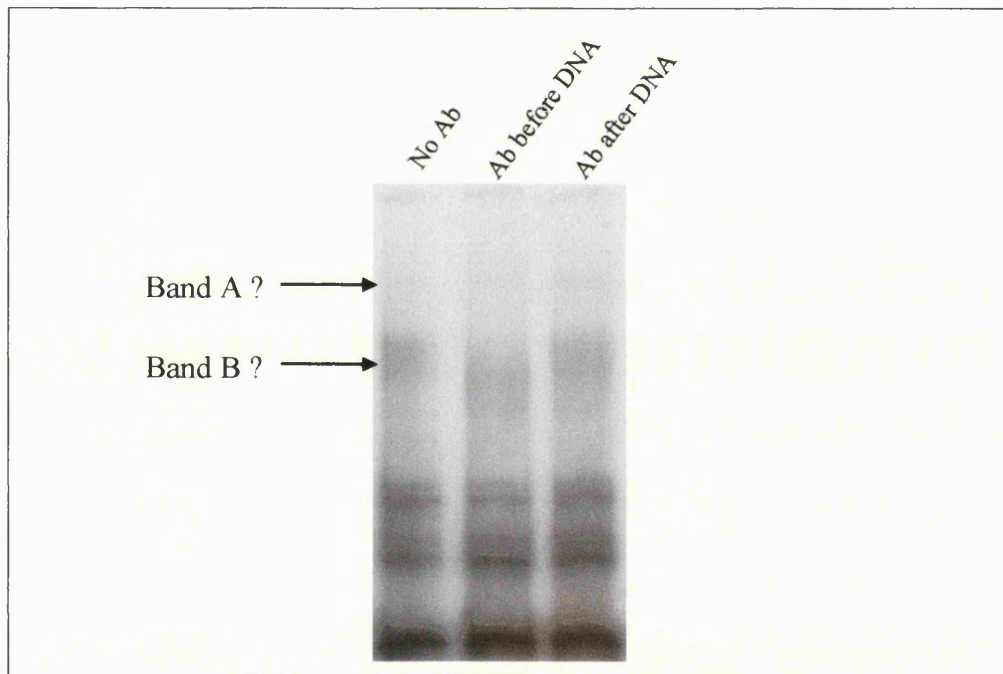
Figure 4.4.9. Supershift assays using SAA2 5' flanking region fragment, HepG2 nuclear
extracts and polyclonal antibodies to YY1. The region where the bands corresponding to
previously identified protein DNA complexes are labelled A and B.

4.5. Sequence analysis of human SAA4

SAA4 differs from SAA1 and SAA2 primarily in that it is not considered to be an acute phase protein and is therefore termed the constitutively expressed SAA isoform. This is not however strictly true. The circulating levels of SAA4 do increase during an acute phase response but only by approximately 5 to 6 fold as opposed to the much larger increases, up to 1000 fold observed for SAA1 and SAA2. Hence SAA4 also appears to be cytokine responsive. Only 130bp of the 5' flanking region of the human SAA4 sequence was available for analysis at the initiation of this study. A further 265bp was sequenced in order to analyse the sequence for possible transcription factor binding sites and for comparison with SAA1 and SAA2. Of all the human SAA genes SAA4 is the least well characterised with regard to transcriptional control mechanisms. The mechanisms determining its basal expression and the increase observed during an acute phase response had not been determined.

The SAA4 5' flanking region extending to position -395bp was sequenced and analysed for possible transcription factor binding sites. The results show that there is a putative C/EBP binding site but no NF-κB binding site was detected (fig 4.5.1). This could explain why SAA4 can be induced but not to the level observed for SAA1 and SAA2. SAA1 and SAA2 are responsive to both IL-1 and IL-6 but it is the synergistic action of the transcription factors NF-κB and C/EBP which is responsible for the high degree of inducibility of the gene which exceeds the additive effect of each cytokine taken individually. The reported increase in SAA4 expression of 6 fold is similar to the fold increase reported for the SAA2 gene in response to IL-6 only.

Figure 4.5.2. SAA4 reporter construct expression levels in response to various stimuli when transfected into HepG2 cells. Results are expressed as an induction ratio.

Figure 4.5.3. SAA4 reporter construct expression levels in response to various stimuli when transfected into HeLa cells. Results are expressed and an induction ratio.

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                                                              -350
CAAACATCTCTTTTGCTAATAATATTGATCACTTCCTACAGCATAATATCTATGTGCAAT
-----+---------+---------+---------+---------+---------+----
GTTTGTAGAGAAACGATTATTATAACTAGTGAAGGATGTCGTATTATAGATACACGTTA

                                              -300
TTTTTGGGAAGACCTCCTACTAAACAGTTAAGAATGGAAAGACACAAAGAGAAGAAAGTG
-----+---------+---------+---------+---------+---------+----
AAAAACCCTTCTGGAGGATGATTTGTCAATTCTTACCTTTCTGTGTTTCTCTTCTTTCAC

                                   -250
GAGAAAGACCTCTGTCTTCCCACACTGGATTTTGCAAATACAACTCTTCTGTGATACAGG
-----+---------+---------+---------+---------+---------+----
CTCTTTCTGGAGACAGAAGGGTGTGACCTAAAACGTTTATGTTGAGAAGACACTATGTCC
                                           HNF-5
              -200
AAACTAAAAAGAGGGAAAAGTGGCAATTAAGAAAAACTGCCAGAAAATAAATACCTTGAT
-----+---------+---------+---------+---------+---------+----
TTTGATTTTTCTCCCTTTTCACCGTTAATTCTTTTTGACGGTCTTTTATTTATGGAACTA

   -150                    XbaI                          -100
GACCCAATTTGGAAAACTACTTCTAGATAAGGAAACACTATTACCCAATATGTTAATTTT
-----+---------+---------+---------+---------+---------+----
CTGGGTTAAACCTTTTGATGAAGATCTATTCCTTTGTGATAATGGGTTATACAATTAAAA

                            C\EBP               -50
TAAAATACCCAAAGAATTGAGCAAAAAGGACTTTGGCAAGGCAGGTTGTTGGATTTAAAA
-----+---------+---------+---------+---------+---------+----
ATTTTATGGGTTTCTTAACTCGTTTTTCCTGAAACCGTTCCGTCCAACAACCTAAATTTT

                                          KpnI
CCGTTCTCCCATTAATGCCAAAGCCAGCAGAGGTACCAACTATAGCTCCACGGCCAGAAG
-----+---------+---------+---------+----------+----- EXON 1 ----------
GGCAAGAGGGTAATTACGGTTTCGGTCGTCTCCATGTTGATATCGAGGTGCCGGTCTTC
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Figure 4.5.1. Sequence and putative transcription factor binding sites indentified in the human SAA4 5' flanking region. Bold type represents region sequenced during this study; restriction sites are shown in red; transcription factor sites in blue; and the exon one sequence in green type.

To investigate if this region of the SAA4 5' flanking region was cytokine responsive a fragment of the SAA4 gene from 0 to -132bp was cloned into the pGL2-basic vector. This reporter construct was transfected into both HepG2 (fig 4.5.2) and HeLa (4.5.3) cell lines which were then stimulated with the cytokines IL-1 and IL-6, both individually and together, LPS and dexamethasone both alone and together with IL-1 and IL-6. The results show that this construct is inducible when transfected into both HepG2 and HeLa cells. However. The response to each of the cytokines IL-1 and IL-6 individually is higher in HepG2 than in HeLa cells. Similar levels of expressionare observed with a combination of IL-1 and IL-6. IL-1 and IL-6 with dexamathasone gives the greatest levels of induction in HeLa cells. These results indicate that if a regulatory element is present within the first

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150 bp of the human SAA4 5' flanking region then it is responsive in both HepG2 and HeLa cells. With some cell specific variation.

5.0.Discussion

5.1. Tissue Distribution of SAA expression

The expression of acute phase proteins is predominantly liver specific. Due to the acute phase nature of the SAA family of proteins the expression of their corresponding genes was also considered to be restricted to the liver. However in recent years there have been a number of published reports of extra hepatic expression of SAA (Ureli-Shovel *et al*, 1994., Meek and Benditt, 1996., Meek and Benditt, 1998), including reports of human SAA expression in non-hepatic cell lines (Steel *et al*, 1996). The question of exactly where SAA is expressed is important in terms of both function and pathology. Extra hepatic expression of human SAA has been predominantly found using immortalised cell lines which could give false and misleading results. Therefore the expression of the human SAA genes was examined in 50 human tissues by dot blot analysis to determine the tissue expression pattern of SAA. The results showed that all tissues examined expressed the acute phase and constitutive isoforms to some degree. However the levels of expression in the liver were significantly higher than any other tissue indicating that SAA does exhibit some degree of liver specific expression. The finding of extra-hepatic expression of SAA has led to speculations as to whether it is locally produced SAA which is cleaved to form the fibrillar constituent in reactive amyloidosis or the hepatically produced systemic SAA protein.

The tissue samples analysed for SAA expression included a number of foetal tissue samples. The expression patterns for the foetal tissues were found to be similar in that the level of expression in the liver was significantly higher than in any other tissue

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examined. The mechanisms determining liver specific expression are therefore present prior to birth. The levels of expression for all foetal tissues were however lower than for the adult/adolescent tissues perhaps indicating that not all of the regulatory control mechanisms were fully functional or present in the foetus. As SAA is acute phase in nature this could possibly relate to the development of the acute phase response and the stage at which the mechanisms involved in the initiation and control of the acute phase response develop.

As has been previously mentioned all tissues examined expressed SAA, this includes brain tissue. In addition to whole brain tissue, SAA expression was also analysed in various individual brain region tissues. SAA was found to be expressed in all regions examined with some regional variations. Reports of extra-hepatic expression of SAA include detection of SAA expression in the brain tissue from patients with Alziehmers disease (Liang *et al*, 1997). A characteristic of Alzheimers Disease is the presence of amyloidotic plaques within the brain. One of the components of these plaques is β-amyloid protein. β-amyloid is formed from a larger precursor protein known as amyloid precursor protein (APP). This shows similarities to secondary or reactive amyloidosis and the cleavage of SAA to the fibrillar constituent AA. The discovery of the presence of SAA is not the first association of acute phase proteins with Alzheimers Disease plaques . Both α-1 antitrypsin (Abraham *et al*, 1988) and α_2-macroglobulin (Strauss *et al*, 1992) have been found associated with the plaques as have the complement components C1q, C3 and C4 (Eikenboom and Stam, 1982), and the cell adhesion molecule ICAM-1(Verbeek *et al*, 1994). The suggestion from these findings that the inflammatory response is in some way associated with the formation of the senile plaques in Alzheimer Disease is supported by the finding that non-steroidal anti-inflammatory agents may prevent or retard the process of Alzheimers Disease (Breitner, 1996).

It is now accepted that SAA is expressed extra-hepatically. Whether this extra-hepatic expression has any functional significance has yet to be elucidated. The finding of extra-hepatic expression alone is not enough to show that this expression is significant, or at high enough levels to be functionally important. The function of SAA has yet to be defined but it may play some role in the immune response or wound healing which would fit with the increase in expression observed during an acute phase response. If so then it is possible that the extra-hepatic expression does serve some functional purpose being produced locally close to sites of injury in addition to the systemic production by the liver. Until the function of SAA has been elucidated it is impossible to say if this is indeed the case, therefore these findings of extra-hepatic expression should not detract from the fact that the liver is the predominant site of expression and hence undoubtedly the most important site of expression.

5.2. Mechanisms of liver specific expression of the human SAA2 gene

The expression of other genes which exhibit liver specificity has been found to be attributable to the cumulative effect of a number of transcription factors which are predominantly, but not exclusively, expressed in the liver. These factors are all found to show some degree of extra-hepatic expression although the pattern of extra-hepatic expression differs for each factor. The presence of a binding site for just one of these factors would not confer tissue specificity to a gene but the presence of multiple sites enables higher levels of expression in the liver than any other tissue as in is the only tissue in which all these 'liver enriched ' factors are expressed. This means that findings of extra-hepatic expression of what are considered to be liver specific proteins are not surprising. The findings of extra-hepatic expression of SAA at significantly lower levels

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than the liver correlates with the mechanisms of liver specific expression reported for other genes suggesting that these same mechanisms may determine the tissue specificity of SAA.

Characterisation of the SAA2 5' flanking region to identify transcriptional control elements had already shown the presence of three cytokine responsive elements. These are two IL-1 responsive NF-κB sites and an IL-6 responsive C/EBP (Edbrooke *et al*, 1989, Edbrooke *et al*, 1991). C/EBP has been implicated in the liver specific other acute phase genes including α-1antitrypsin and transthyretin and is one of the group of transcription factors known as 'liver enriched' factors. The region of the SAA2 5' flanking region containing the C/EBP binding site was analysed to determine if it had tissue specific properties by transient transfection assays using HepG2 and HeLa cell lines. There was a difference in the expression pattern observed for the two cell lines. The presence of the C/EBP site in reporter constructs results in a greater level of expression due to increased cytokine reponsiveness. This coincides with the implication of C/EBP in liver specific expression and it's involvement in the transcriptional regulation of other liver specific genes. C/EBP itself is a family of transcription factors. It has been found that C/EBPβ and C/EBPδ but not C/EBPα bind to the C/EBP site of the SAA2 5' flanking region in response to IL-6 stimulation. C/EBPβ which was originally termed NF-IL-6 but has since been renamed. This factor is predominantly expressed in the liver and kidney but is also found in a number of other tissues. C/EBPδ is also found in a number of tissues but is predominantly expressed in the lung.

Transfection studies identified a 140 bp region of the SAA2 5' flanking region with tissue specific regulatory properties. This region appears to contain an element which repressed cytokine induced expression of the SAA2 in non-hepatic cells. This fragment of the

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SAA2 gene was also found to have protein binding properties forming two complexes. The protein component of one of these complexes appears to be ubiquitously expressed while the other is liver specific. The results are surprising in that these protein components also appear to be constitutively expressed and not produced in response to cytokine stimulation. No additional complexes were formed in response to cytokine stimulation. The results from the transient transfection assays had shown the tissue specificity to be cytokine induced. Absence of a band corresponding to a cytokine induced DNA-protein complex does not conclusively prove that a complex is not being formed. Formation of a protein DNA complex can be influenced by a number of factors the most notable of which is salt concentration with different factors preferentially binding under different conditions. Although binding of protein to DNA may occur intra cellularly as in transfection studies. The conditions for binding outside of the cellular enviroment as in EMSAs may not be optimal.

Sequence analysis of the -2213bp to -2255bp region of the SAA2 gene to identify known transcription factor binding sites failed to identify possible binding sites for any of the 'liver enriched' factors. A number of putative transcription factor binding sites were identified, of particular interest were possible sites for the transcription factors YY1 and AP-2. Mutational analysis described in this thesis revealed that mutation of the putative YY1 site did have an effect on formation of the ubiquitous complex. It did not prevent formation of a complex but appeared to cause formation of a smaller complex. Use of antibodies to YY1 to confirm that it is the factor YY1 which is binding to the DNA were inconclusive and further assays will need to be carried out in order to identify this DNA binding protein. In addition competition assays using an oligonucleotide known to bind the factor YY1 would also be of use in identifying the DNA binding protein. If YY1 does bind to the region human SAA2 being studied than such an oligonucleotide , which is

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documented as binding the transcription factor YY1 should compete with SAA2 for binding of the factor. If such competition was seen then this factor is almost certainly YY1 or a YY1 like factor. If further studies confirm the identity of this protein as YY1 then this suggest that YY1 is part of a complex which binds to the SAA2 5' flanking region along with another as yet unidentified protein component.

YY1 has been reported to be involved in transcriptional control of the rat SAA1 gene. YY1 binds to a region within the rat SAA1 5' flanking region which overlaps the binding sites for NF-κB and C/EBP which binds the factor (Lu et al, 1994). YY1 binds to this region to actively repress SAA1 expression in the uninduced state causing low basal levels of SAA1. Upon stimulation with conditioned media YY1 was displaced from it's binding site, NF-κB and C/EBP bind to their respective binding sites which were being blocked by YY1 and activate expression of the SAA1 gene. YY1 is a ubiquitously expressed transcription factor capable of both activation and repression. YY1 can alter transcriptional activity by binding directly to DNA, interacting with other transcription factors binding to the DNA or by binding to transcription factors and preventing them from binding to DNA, it has also been found to cause DNA bending which may serve to bring other bound transcription factors into close proximity to each other.

It has been well documented that YY1 can interact with numerous other transcription factors (see section 1.5.4), one such factor is C/EBPβ . YY1 and C/EBPβ have been found in complex on the HPV-18 upstream regulatory region. YY1 is known to have both activating and repressing properties and in the HPV-18 promoter has been found to have both. In HepG2 cells YY1 acts as a repressor of HPV-18 transcription. In HeLa cells YY1 is a positive activator of HPV-18 transcription provided an upstream sequence termed the 'switch region' is present. This region has been found to be bound by

C/EBPβ. When the switch region is deleted or mutated, YY1 acts as a repressor of HPV-18 expression in HeLa cells.

There is also evidence to suggest that YY1 may interact with the transcription factor AP-2. AP-2 is a transcription factor which was first was originally identified in HeLa cells. This original factor has since been renamed AP-2α when it was found that there are actually a family of AP-2 proteins. To date two further members of this family have been identified and named AP-2β and AP-2γ. The AP-2 transcription factors exhibit tissue specificity in their expression. The original factor, AP-2α, is found in HeLa and fibroblast cells, but not in HepG2 cells. AP-2α is a negative regulator of transcriptional activation. It's activity has been found to be induced in response to phorbol esters and signals that enhance cyclic AMP levels. This occurs independently of *de novo* protein synthesis (Lusher *et al*, 1989., Buetner *et al*, 1993).

YY1 has been found to be a repressor of transcriptional activity of the human gamma interferon gene. However YY1 alone is not sufficient for the repression of the gene but requires binding of a second transcription factor to the silencer element. This factor has been identified as an AP-2-like transcription factor. It a recent review on the subject of the transcription factor YY1 it was noted that almost all of those genes which have been identified as being targets of YY1 repressional activity are cytokine induced or are associated with differentiation.

The tissue specific element identified within the SAA2 5' flanking region does not occur in the region which shares a high degree of homology with SAA1. It is therefore unlikely that a similar element will be present at the same position in the SAA1 5' flanking region. If present such an element is perhaps more likely to be situated at a different location in

which case the studies carried out to identify this region within the SAA2 gene would need to be repeated using SAA1 reporter constructs. It is also possible that the control of SAA1 liver specific expression involves mechanisms different to those identified for SAA2.

In addition to the region of the SAA2 gene shown to have tissue specific properties, the entire SAA2 5' flanking region from +35 bp to -2213 was also analysed for possible transcription factor binding sites (data not shown). These analyses did show a number of possible sites for the liver enriched transcription factors however sequence analysis alone is not evidence of a transcription factor binding site. This would need to be confirmed by functional expression studies using reporter constructs and by DNA-protein binding assays. As mentioned previously, because of the mechanisms by which liver specificity is conferred the use of a wider range of cell lines may identify some of these regions as having tissue specific properties.

Two transgenic mouse lines have been created within this laboratory by David Faulkes which contain the entire human SAA2 coding region and either 440bp (440-SAA2) or 4500bp (4500-SAA2) of SAA2 5' flanking region. Human SAA2 expression was analysed in a number of tissues subsequent to stimulation with LPS. For the 440-SAA2 transgenic mice human SAA2 expression was found in liver, kidney and adipose tissues at similar levels. Low levels of expression were also found in brain and spleen. Human SAA2 expression in the 4500-SAA2 transgenic mice was only found in high levels in the liver. Low levels of expression were found in adipose and kidney tissue with no expression detectable in spleen and brain. These results indicate the presence of an element with tissues specific properties 5' of the -440bp region of the gene at which the

homology with human SAA1 breaks down and correlate with the findings in this thesis.

The identity of the proteins binding to the -2213bp to -2355bp region of the human SAA2 5' flanking region has yet to be confirmed. This needs to be done with the use of polyclonal antibodies to the candidate transcription factors mentioned. This work had been initiated using an antibody to YY1 but the results are preliminary and need to be repeated. An antibody to the factor AP-2α has also been acquired and will be used to determine if this factor is a component of the DNA-protein complexes. Antibodies to the liver enriched transcription factors are now commercially available and further supershift analysis of the liver specific protein DNA complex using these antibodies should be carried out. Although no such liver enriched transcription factor binding sites were identified by sequence analysis this does no rule out the possibility that such a site is present. In addition C/EBP family antibodies are currently available within the laboratory although it seems unlikely that C/EBP is part of the complexes as C/EBP is an IL-1 and IL-6 induced transcription factor and the protein-DNA complexes were not formed in response to cytokine stimulation. The possibility exists that one or more proteins which interact with the DNA may not be one of those factors mentioned above or may even be an as of yet unidentified factor. In order to gain more information as to the actual binding sites of these proteins methylation interference assays have been carried out to try and pin point the DNA bases with which the proteins interact. These studies are continuing. Once these bases have been identified, mutational analysis can be used to confirm the functional properties of these sites and their importance in the liver specific expression of SAA.

The transient transfection studies also revealed that the C/EBP site which had been previously identified within the SAA2 5' flanking region exhibited tissue specific

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regulation. As C/EBP family members which bind this site are known to be liver enriched in their expression then this result is not surprising. This factor should also be analysed in more detail as to it's role in the tissue specific expression of SAA by analysing DNA-protein complex formation in both hepatic and non-hepatic cell lines and subsequent functional mutation analysis. This element is particularly interesting as it is also present in the SAA1 5' flanking region

5.3. Expression and Transcriptional Regulation of Human SAA4

Analysis of SAA4 expression using a multiple human tissue dot blot revealed that like SAA1/SAA2 SAA4 expression could be detected in all tissues tested but that the level of expression in the liver was significantly higher than in any other tissue. The difference between the hepatic and non-hepatic expression levels was not however as great as for SAA1/SAA2 expression. SAA4 is the least well characterised of the SAA family with regards to transcriptional control elements. No transcriptional control elements had been identified at the initiation of this work. Sequence analysis identified the presence of potential binding sites for a number of transcription factors the most notable of which is C/EBP. As has previously been discussed C/EBP is a family of transcription factors. This C/EBP site my bind a member of the family which is present in both cell lines. Alternately, this set of transfection experiments was carried out under the conditions previously utilised within this laboratory with the use of CAT reporter constructs. The transfected cells were harvested 16 hours after stimulation with IL-6 etc. Although SAA4 is known as the constitutive human SAA isoform circulating levels of the protein do increase during an acute phase response by about 6 fold. Therefore the time at which the cells were harvested for assaying may have affected the results.

The increase in the circulating levels of the acute phase SAA proteins, which can be up to 1000 fold, has been attributed a synergistic response to the cytokines IL-1 and IL-6. It has been found *in vitro* that the response of SAA2 to a combination of IL-1 and IL-6 is significantly higher than the additive response to each cytokine taken individually. The transcription factors C/EBP and NF-κB have been found to interact to produce a synergistic transcriptional activation which is responsible for the high inducibilty of the gene. The absence of one of these factors would result in a significantly lower level of inducibilty. It is possible that this is the case with SAA4 and that the acute phase or constitutive property of the human SAA genes is conferred by the presence or absence of a binding site for the transcription factor NF-κB.

Analysis of the transcriptional control mechanisms of human SAA4 is still in the early stages. These mechanisms need to fully elucidated and understood before the involvement of these or other factors can be further investigated. There is very little sequence homology between the 5' regulatory regions of the human SAA4 gene and the two acute phase isoform genes. It is unlikely that if the mechanisms of tissue specific expression of this gene resemble those of SAA2 that they will be in the same location. In addition to the difference in inducibility of the gene human SAA4 also differs from SAA1 and SAA2 in that the circulating levels in the absence of an acute phase response are considerably higher at 15-20mg/ml of blood as opposed to 1-2mg/ml for SAA1/SAA2 combined.

Tissue specific expression of SAA appears, from this study, to coincide with the mechanisms determined for other liver specific genes in that it involves multiple factors. One of these factors being C/EBP. A second tissue specific element has been identified within the SAA2 gene which binds multiple nuclear factors. One of these factors is an

as of yet unidentified liver specific protein. These studies have indicated that a second

protein binding this element could be the transcription factor YY1.

Appendix 1   References

Abraham-CR, Selkoe-DJ, and Potter-H (1988) Immunochemical identification of the serine protease inhibitor alpha 1-antichymotrypsin in the brain amyloid deposits of Alzheimer's disease. *Cell.* **52** 487-501 .

Agarwal-KL, and Riftina-F   (1979) Synthesis and enzymatic properties of deoxyribo-oligonucleotides containing methyl and phenylphosphonate linkages. *Nucleic. Acids. Res* **6** 3009-3024 .

Aggarwal-BB, Kohr-WJ, Hass-PE, Moffat-B, Spencer-SA, Henzel-WJ, Bringman-TS, Nedwin-GE, Goeddel-DV, and Harkins-RN (1985) Human tumor necrosis factor. Production, purification, and characterization. *J. Biol. Chem.* **260** 2345-2354 .

Akira-S, Isshiki-H, Sugita-T, Tanabe-O, Kinoshita-S, Nishio-Y, Nakajima-T, Hirano-T, and Kishimoto-T (1990) A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO. J.* **9** 1897-1906 .

Akira-S, Nishio-Y, Inoue-M, Wang-XJ, Wei-S, Matsusaka-T, Yoshida-K, Sudo-T, Naruto-M, and Kishimoto-T (1994) Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. *Cell.* **77** 63-71.

Antonson-P, and Xanthopoulos-KG (1995) Molecular cloning, sequence, and expression patterns of the human gene encoding CCAAT/enhancer binding protein alpha (C/EBP alpha). *Biochem. Biophys. Res. Commun.* **215** 106-113 .

Arend-WP (1991) Interleukin 1 receptor antagonist. A new member of the interleukin 1 family.

J. Clin. Invest. **88** 1445-1451.

Arend-WP, Joslin-FG, and Massoni-RJ (1985) Characteristics of chondrocyte responses to a human interleukin 1-like factor. *Clin. Immunol. Immunopathol.* **36** 358-370.

Auron-PE, Webb-AC, Rosenwasser-LJ, Mucci-SF, Rich-A, Wolff-SM, and Dinarello-CA (1984) Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. *Proc. Natl. Acad. Sci. U. S. A.* **81** 7907-7911.

Bach-I, Mattei-M-G, Cereghini-S, and Yaniv-M (1991) Two members of an HNF1 homeoprotein family are expressed in human liver *Nucleic. Acids. Res.* **19** 3553-3559.

Badolato-R, Wang-JM, Murphy-WJ, Lloyd-AR, Michiel-DF, Bausserman-LL, Kelvin-DJ, and Oppenheim-JJ (1994) Serum amyloid A is a chemoattractant: induction of migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes. *J. Exp. Med.* **180** 203-209.

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

Baeuerle-PA, and Henkel-T (1994) Function and activation of NF-kappa B in the immune system. *Ann. Rev. Immunol* **12** 141-179 .

Baltz-ML, Caspi-D, Evans-DJ, Rowe-IF, Hind-CR, and Pepys-MB (1986) Circulating serum amyloid P component is the precursor of amyloid P component in tissue amyloid deposits. *Clin. Exp. Immunol.* **66** 691-700 .

Bankers-Fulbright-JL, Kalli-KR, and McKean-DJ (1996) Interleukin-1 signal transduction. *Life. Sci.* **59** 61-83.

Baumann-G, Geisse-S, and Sullivan-M (1991) Cyclosporin A and FK-506 both affect DNA binding of regulatory nuclear proteins to the human interleukin-2 promoter. *New. Biol.* **3** 270-278 .

Baumann-H, and Gauldie-J (1994) The acute phase response *Immunol. Today.* **15** 74-80.

Baumhueter-S, Courtois-G, Morgan-JG, and Crabtree-GR (1989) The role of HNF-1 in liver-specific gene expression. *Ann. N. Y. Acad. Sci* **557** 272-278, discussion 279 .

Bazan-JF (1991) Neuropoietic cytokines in the hematopoietic fold. *Neuron.* **7** 197-208.

Beg-AA, Ruben-SM, Scheinman-RI, Haskill-S, Rosen-CA, and Baldwin-AS Jr (1992) I kappa B interacts with the nuclear localization sequences of the subunits of NF-kappa B: a mechanism for cytoplasmic retention *Genes. Dev.* **6** 1899-1913 .

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

Betts-JC, Cheshire-JK, Akira-S, Kishimoto-T, and Woo-P (1993) The role of NF-kappa B and NF-IL6 transactivating factors in the synergistic activation of human serum amyloid A gene expression by interleukin-1 and interleukin-6. *J. Biol. Chem.* **268** 25624-25631.

Betts-JC, Edbrooke-MR, Thakker-RV, and Woo-P (1991) The human acute-phase serum amyloid A gene family: structure, evolution and expression in hepatoma cells. *Scand. J. Immunol.* **34** 471-482.

Beutler-B (1992) Application of transcriptional and posttranscriptional reporter constructs to the analysis of tumor necrosis factor gene regulation. *Am. J. Med. Sci.* **303** 129-133.

Bhardwaj-N, Santhanam-U, Lau-LL, Tatter-SB, Ghrayeb-J, Rivelis-M, Steinman-RM, Sehgal-PB, and May-LT (1989) IL-6/IFN-beta 2 in synovial effusions of patients with rheumatoid arthritis and other arthritides. Identification of several isoforms and studies of cellular sources. *J. Immunol.* **143** 2153-2159.

Birnboim-HC, and Doly-J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA *Nucleic. Acids. Res* **7** 1513.

Blumenfeld-M, Maury-M, Chouard-T, Yaniv-M, and Condamine-H (1991) Hepatic Nuclear Factor 1 (HNF1) shows a wider distribution than products of its known target genes in developing mouse *Development* **113** 589-599.

Breitner-JC (1996) The role of anti-inflammatory drugs in the prevention and treatment of Alzheimer's disease. *Ann. Rev. Med* **47** 401-411 .

Bruce-AG, Linsley-PS, and Rose-TM (1992) Oncostatin M. *Prog. Growth. Factor. Res.* **4** 157-170.

Buchta-R, Fridkin-M, Pontet-M, Contessi-E, Scaggiante-B, and Romeo-D (1987) Modulation of human neutrophil function by C-reactive protein. *Eur. J. Biochem.* **163** 141-146 .

Burstein-SA, Mei-RL, Henthorn-J, Friese-P, and Turner-K (1992) Leukemia inhibitory factor and interleukin-11 promote maturation of murine and human megakaryocytes in vitro. *J. Cell. Physiol.* **153** 305-312.

Bushmeyer-S, Park-K, and Atchison-ML (1995) Characterization of functional domains within the multifunctional transcription factor, YY1. *J. Biol. Chem.* **270** 30213-30220 .

Cao-Z, Umek-RM, and McKnight-SL (1991) Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes. Dev.* **5** 1538-1552 .

Cereghini-S, Blumenfeld-M, and Yaniv-M (1988) A liver-specific factor essential for albumin transcription differs between differentiated and dedifferentiated rat hepatoma cells. *Genes. Dev* **2** 957-974 .

Chedid-M, and 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, and Mizel-SB (1989) Signal transduction pathway for IL-1. Involvement of a pertussis toxin-sensitive GTP-binding protein in the activation of adenylate cyclase. *J. Immunol* **142** 4301-4306 .

Chouard-T, Blumenfeld-M, Bach-I, Vandekerckhove-J, Cereghini-S, and Yaniv-M (1990) A distal dimerization domain is essential for DNA-binding by the atypical HNF1 homeodomain *Nucleic. Acids. Res.* **18** 5853-5863.

Claus-DR, Siegel-J, Petras-K, Skor-D, Osmand-AP, and Gewurz-H (1977) Complement activation by interaction of polyanions and polycations. III. Complement activation by interaction of multiple polyanious and polycations is the presence of C-reactive protein. *J. Immunol.* **118** 83-87.

Cooper-JH (1974) Selective amyloid staining as a function of amyloid composition and structure. Histochemical analysis of the alkaline Congo red, standardized toluidine blue, and

iodine methods. *Lab. Invest.* **31** 232-238.

Costa-RH, Grayson-DR, and Darnell-JE Jr (1989) Multiple hepatocyte-enriched nuclear factors function in the regulation of transthyretin and alpha 1-antitrypsin genes. *Mol. Cell. Biol.* **9** 1415-1425 .

Costa-RH, Van-Dyke-TA, Yan-C, Kuo-F, and Darnell-JE Jr (1990) Similarities in transthyretin gene expression and differences in transcription factors: liver and yolk sac compared to choroid plexus. *Proc. Natl. Acad. Sci. U. S. A.* **87** 6589-6593 .

Croston-GE, Cao-Z, and Goeddel-DV (1995) NF-kappa B activation by interleukin-1 (IL-1) requires an IL-1 receptor-associated protein kinase activity. *J. Biol. Chem* **270** 16514-16517
.

Davis-S, Aldrich-TH, Stahl-N, Pan-L, Taga-T, Kishimoto-T, Ip-NY, and Yancopoulos-GD (1993) LIFR beta and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor. *Science.* **260** 1805-1808.

deBeer-MC, deBeer-FC, Gerardot-CJ, Darin-CR, Webb-NR, Goodison-ML, and Kindy-MS (1996) Structure of the mouse SAA4 gene and its linkage to the SAA gene family *Genomics* **34** 139-142.

Dembic-Z, Loetscher-H, Gubler-U, Pan-YC, Lahm-HW, Gentz-R, Brockhaus-M, and Lesslauer-W (1990) Two human TNF receptors have similar extracellular, but distinct intracellular, domain sequences. *Cytokine.* **2** 231-237.

De-Simone-V, Ciliberto-G, Hardon-E, Paonessa-G, Palla-F, Lundberg-L, and Cortese-R (1987) Cis- and trans-acting elements responsible for the cell-specific expression of the human alpha 1-antitrypsin gene. *EMBO. J.* **6** 2759-2766 .

De-Simone-V, and Cortese-R (1992) Transcription factors and liver-specific genes. *Biochim. Biophys. Acta.* **1132** 119-126 .

De-Simone-V, De-Magistris-L, Lazzaro-D, Gerstner-J, Monaci-P, Nicosia-A, and Cortese-R (1991) LFB3, a heterodimer-forming homeoprotein of the LFB1 family, is expressed in specialized epithelia *EMBO. J.* **10** 1435-1443.

de-Wet-JR, Wood-KV, DeLuca-M, Helinski-DR, and Subramani-S (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* **7** 725-737.

Dickson-PW, Howlett-GJ, and Schreiber-G (1982) Metabolism of prealbumin in rats and changes induced by acute inflammation. *Eur-J-Biochem.* **129** 289-293.

Dignam-JD (1990) Preparation of extracts from higher eukaryotes. *Methods. Enzymol.* **182** 194-203.

Doppler-W, Welte-T, and Philipp-S (1995) CCAAT/enhancer-binding protein isoforms beta and delta are expressed in mammary epithelial cells and bind to multiple sites in the beta-casein gene promoter. *J. Biol. Chem.* **270** 17962-17969 .

Duff-G (1985) Immune diseases. Many roles for interleukin-1. *Nature* **313** 352-353 .

Dustin-ML, and Springer-TA (1991) Role of lymphocyte adhesion receptors in transient

interactions and cell locomotion. *Annu-Rev-Immunol.* **9** 27-66.

Eck-MJ, and Sprang-SR (1989) The structure of tumor necrosis factor-alpha at 2.6 A resolution. Implications for receptor binding. *J. Biol. Chem.* **264** 17595-17605 .

Edbrooke-MR, Burt-DW, Cheshire-JK, and Woo-P (1989) Identification of cis-acting sequences responsible for phorbol ester induction of human serum amyloid A gene expression via a nuclear factor kappaB-like transcription factor. *Mol. Cell. Biol.* **9** 1908-1919.

Edbrooke-MR, Foldi-J, Cheshire-JK, Li-F, Faulkes-DJ, and Woo-P (1991) Constitutive and NF-kappa B-like proteins in the regulation of the serum amyloid A gene by interleukin 1. *Cytokine.* **3** 380-388.

Eikelenboom-P, and Stam-FC (1982) Immunoglobulins and complement factors in senile plaques. An immunoperoxidase study. *Acta. Neuropathol. Berl.* **57** 239-242 .

Faulkes-DJ, Betts-JC, and Woo-P (1994) Characterization of five human serum amyloid A1 alleles *Amyloid: Int. J. Exp. Clin. Invest.* **1** 255-262.

Faulkes-DJ, Betts-JC, and Woo-P (1997) Do alleles at the serum amylooid A locus influence susceptibility to reactive amyloidosis in systemic onset juvenile chronic arthritis *Amyloid: Int. J. Exp. Clin. Invest.* **4** 75-79.

Floyd-Smith-G, Whitehead-AS, Colten-HR, and Francke-U (1986) The human C-reactive protein gene (CRP) and serum amyloid P component gene (APCS) are located on the proximal long arm of chromosome 1. *Immunogenetics* **24** 171-176 .

Friedman-B, Scherer-SS, Rudge-JS, Helgren-M, Morrisey-D, McClain-J, Wang-DY, Wiegand-

SJ, Furth-ME, Lindsay-RM, and et-al (1992) Regulation of ciliary neurotrophic factor expression in myelin-related Schwann cells in vivo. *Neuron.* **9** 295-305 .

Fuchs-P, Strehl-S, Dworzak-M, Himmler-A, and Ambros-PF (1992) Structure of the human TNF receptor 1 (p60) gene (TNFR1) and localization to chromosome 12p13 *Genomics.* **13** 219-224.

Fyfe-AI, Rothenberg-LS, DeBeer-FC, Cantor-RM, Rotter-JI, and Lusis-AJ (1997) Association between serum amyloid A proteins and coronary artery disease: evidence from two distinct arteriosclerotic processes. *Circulation.* **96** 2914-2919.

Garner-MM, and Revzin-A (1981) A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the Escherichia coli lactose operon regulatory system. *Nucleic. Acids. Res.* **9** 3047-3060 .

Gearing-DP, and Bruce-AG (1992) Oncostatin M binds the high-affinity leukemia inhibitory factor receptor. *New. Biol.* **4** 61-65.

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

Grabstein-KH, Baker-PE, Hess-B, Clark-L, Slack-J, Sims-JE, Alpert-AR, Dower-S, Weisser-KE, and Overell-RW (1992) Regulation of murine B-cell growth in vitro by infection with a retroviral vector containing the murine IL-1R. *Ann. N. Y. Acad. Sci.* **651** 474-476.

Graham-FL, and Eb-AJ-van-der (1973) Transformation of rat cells by DNA of human adenovirus 5. *Virology.* **54** 536-539 .

145

Grange-T, Roux-J, Rigaud-G, and Pictet-R (1991) Cell-type specific activity of two glucocorticoid responsive units of rat tyrosine aminotransferase gene is associated with multiple binding sites for C/EBP and a novel liver-specific nuclear factor. *Nucleic. Acids. Res.* **19** 131-139 .

Grayson-DR, Costa-RH, and Darnell-JE (1989) Regulation of hepatocyte-specific gene expression. *Ann. N. Y. Acad. Sci.* **557** 243-55, discussion 255-256 .

Greenfeder-SA, Nunes-P, Kwee-L, Labow-M, Chizzonite-RA, and Ju-G (1995) Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. *J. Biol. Chem.* **270** 13757-13765 .

Guy-GR, Cao-X, Chua-SP, and Tan-YH (1992) Okadaic acid mimics multiple changes in early protein phosphorylation and gene expression induced by tumor necrosis factor or interleukin-1. *J. Biol. Chem.* **267** 1846-1852 .

Haskill-S, Beg-AA, Tompkins-SM, Morris-JS, Yurochko-AD, Sampson-Johannes-A, Mondal-K, Ralph-P, and Baldwin-AS Jr (1991) Characterization of an immediate-early gene induced in adherent monocytes that encodes I kappa B-like activity. *Cell.* **65** 1281-1289.

Hebert-L, and 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 mice. *Scand. J. Immunol.* **31** 167-173.

Henkel-T, Zabel-U, van-Zee-K, Muller-JM, Fanning-E, and Baeuerle-PA (1992) Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-kappa B subunit. *Cell* **68** 1121-1133.

Hibi-M, Murakami-M, Saito-M, Hirano-T, Taga-T, and Kishimoto-T (1990) Molecular

146

cloning and expression of an IL-6 signal transducer, gp130. *Cell.* **63** 1149-1157.

Hirano-T, Matsuda-T, Turner-M, Miyasaka-N, Buchan-G, Tang-B, Sato-K, Shimizu-M, Maini-R, Feldmann-M, and et-al (1988) Excessive production of interleukin 6/B cell stimulatory factor-2 in rheumatoid arthritis. *Eur. J. Immunol.* **18** 1797-1801.

Huang-JH, Reinhoff-HY, and Liao-WS (1994) Regulation of Mouse Serum Amyloid A Gene Expression in Transfected Hepatoma Cells *Mol. Cell. Biol.* **10** 3619-3625.

Ip-NY, and Yancopoulos-GD (1992) Ciliary neurotrophic factor and its receptor complex. *Prog. Growth. Factor. Res.* **4** 139-155 .

Ip-NY, Nye-SH, Boulton-TG, Davis-S, Taga-T, Li-Y, Birren-SJ, Yasukawa-K, Kishimoto-T, Anderson-DJ, and et-al (1992) CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. *Cell* **69** 1121-1132.

Ip-NY, Wiegand-SJ, Morse-J, and Rudge-JS (1993) Injury-induced regulation of ciliary neurotrophic factor mRNA in the adult rat brain. *Eur. J. Neurosci.* **5** 25-33.

Isshiki-H, Akira-S, Sugita-T, Nishio-Y, Hashimoto-S, Pawlowski-T, Suematsu-S, and Kishimoto-T (1991) Reciprocal expression of NF-IL6 and C/EBP in hepatocytes: possible involvement of NF-IL6 in acute phase protein gene expression. *New. Biol.* **3** 63-70 .

Jain-VK, and Magrath-IT (1991) A chemiluminescent assay for quantitation of beta-galactosidase in the femtogram range: application to quantitation of beta-galactosidase in lacZ-transfected cells. *Anal. Biochem.* **199** 119-124.

147

Jobling-SA, Auron-PE, Gurka-G, Webb-AC, McDonald-B, Rosenwasser-LJ, and Gehrke-L (1988) Biological activity and receptor binding of human prointerleukin-1 beta and subpeptides. *J. Biol. Chem.* **263** 16372-16378.

Johnson-PF, Landshultz-WH, Graver-BJ, and 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.

Kawashima-I, Ohsumi-J, Mita-Honjo-K, Shimoda-Takano-K, Ishikawa-H, Sakakibara-S, Miyadai-K, and Takiguchi-Y (1991) Molecular cloning of cDNA encoding adipogenesis inhibitory factor and identity with interleukin-11. *FEBS. Lett.* **283** 199-202.

Kilian-PL, Kaffka-KL, Stern-AS, Woehle-D, Benjamin-WR, Dechiara-TM, Gubler-U, Farrar-JJ, Mizel-SB, and Lomedico-PT (1986) Interleukin 1 alpha and interleukin 1 beta bind to the same receptor on T cells. *J. Immunol* **136** 4509-4514.

Kisilevsky-R, Narindrasorasak-S, Tape-C, and Boudreau-L (1994) During AA amyloidogenesis is proteolytic attack on serum amyloid A a pre- or post-fibrillogenic event *Amyloid:Int. J. Exp. Clin. Invest* **1** 173-183.

Kohno-K, and Kurimoto-M (1998) Interleukin 18, a cytokine which resembles IL-1 structurally and IL-12 functionally but exerts its effects independently of both *Clin Immunol Immunopathol* **86** 11-15.

Kumon-Y, Loose-LD, Birbara-CA, and Sipe-JD (1997) Rheumatoid arthritis exhibits reduced acute phase and enhanced constitutive serum amyloid A protein in synovial fluid relative to serum. A comparison with C-reactive protein. *J. Rheumatol.* **24** 14-19 .

Kuo-CJ, Conley-PB, Chen-L, Sladek-FM, Darnell-JE Jr, and Crabtree-GR (1992) A transcriptional hierarchy involved in mammalian cell-type specification *Nature* **355** 457-461.

Kushner-I (1982) The phenomenon of the acute phase response. *Ann. N. Y. Acad. Sci.* **389** 39-48.

Kushner-I, and Rzewnicki-DL (1994) The acute phase response: general aspects. *Baillieres. Clin. Rheumatol.* **8** 513-530 .

Lai-E, and Darnell-JE Jr (1991) Transcriptional control in hepatocytes: a window on development. *Trends. Biochem. Sci.* **16** 427-430 .

Landschulz-WH, Johnson-PF, and McKnight-SL (1988) The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science.* **240** 1759-1764.

Lazzarro-D, De-Simone-V, De-Magistris-L, Lehtonen-E, and Cortese-R (1992) LFB1 and LFB3 homeoproteins are sequentially expressed during kidney development *Development* **114** 469-479.

Ledebur-HC, and Parks-TP (1995) Transcriptional regulation of the intercellular adhesion molecule-1 gene by inflammatory cytokines in human endothelial cells. Essential roles of a variant NF-kappa B site and p65 homodimers. *J. Biol. Chem.* **270** 933-943.

Lee-JS, See-RH, Galvin-KM, Wang-J, and Shi-Y (1995) Functional interactions between YY1 and adenovirus E1A. *Nucleic. Acid. Res.* **23** 925-931.

Lee-TC, Shi-Y, and Schwartz-RJ (1992) Displacement of BrdUrd-induced YY1 by serum response factor activates skeletal alpha-actin transcription in embryonic myoblasts. *Proc. Natl.*

Acad. Sci. U. S. A. **89** 9814-9818.

Lee-TC, Zhang-Y, and Schwartz-RJ (1994) Bifunctional transcriptional properties of YY1 in regulating muscle actin and c-myc gene expression during myogenesis. *Oncogene* **9** 1047-1052.

Liang-J, Schreiber-BM, Salmona-M, Phillip-G, Gonnerman-WA, deBeer-FC, and Sipe-J (1996) Amino terminal region of acute phase, but not constitutive serum amyloid A (apoSAA) specifically binds and transports cholesterol into aortic smooth muscle and HepG2 cells *Journal of Lipid Research* **37** 1109-1126.

Liang-JS, Sloane-JA, Wells-JM, Abraham-CR, Fine-RE, and Sipe-JD (1997) Evidence for local production of acute phase response apolipoprotein serum amyloid A in Alzheimer's disease brain. *Neurosci. Lett.* **225** 73-76.

Liao-F, Andalibi-A, Lusis-AJ, and Fogelman-AM (1995) Genetic control of the inflammatory response induced by oxidized lipids. *Am. J. Cardiol.* **75** 65B-66B.

Liao-F, Andalibi-A, Qiao-JH, Allayee-H, Fogelman-AM, and Lusis-AJ (1994) Genetic evidence for a common pathway mediating oxidative stress, inflammatory gene induction, and aortic fatty streak formation in mice. *J. Clin. Invest.* **94** 877-884.

Lindhorst-E, Young-D, Bagshaw-W, Hyland-M, and Kisilevsky-R (1997) Acute inflammation, acute phase serum amyloid A and cholesterol metabolism in the mouse. *Biochim. Biophys. Acta.* **1339** 143-154 .

Linke-RP, Bock-V, Valet-G, and Rothe-G (1991) Inhibition of the oxidative burst response of

n-formyl peptide-stimulated neutrophils by serum amyloid A protein *Biochem. Biophys. Res. Com.* **176** 1100-1105.

Li-X, and Liao-WS (1992) Cooperative effects of C/EBP-like and NF kappa B-like binding sites on rat serum amyloid A1 gene expression in liver cells. *Nucleic. Acids. Res.* **20** 4765-4772

.

Li-XX, Huang-JH, Rienhoff-HY Jr, and Liao-WS (1990) Two adjacent C/EBP-binding sequences that participate in the cell-specific expression of the mouse serum amyloid A3 gene. *Moll. Cell. Biol* **10** 6624-6631 .

Li-XX, and Liao-WS (1991) Expression of rat serum amyloid A1 gene involves both C/EBP-like and NF kappa B-like transcription factors. *J. Biol. Chem.* **266** 15192-15201.

Louis-JC, Magal-E, Takayama-S, and Varon-S (1993) CNTF protection of oligodendrocytes against natural and tumor necrosis factor-induced death. *Science.* **259** 689-692.

Lowell-CA, Potter-DA, Stearman-RS, and Morrow-JF (1986) Structure of the murine serum amyloid A gene family. Gene conversion. *J. Biol. Chem.* **261** 8442-8452.

Lu-SY, Rodriguez-M, and Liao-WS (1994) YY1 represses rat serum amyloid A1 gene transcription and is antagonized by NF-kappa B during acute-phase response. *Mol. Cell. Biol.* **14** 6253-6263.

Male-D, Cooke-A, Owen-M, Trowsdale-J, and Champion-B (1996) Cytokines and Chemokines

Malik-N, Kallestad-JC, Gunderson-NL, Austin-SD, Neubauer-MG, Ochs-V, Marquardt-H, Zarling-JM, Shoyab-M, Wei-CM, and et-al (1989) Molecular cloning, sequence analysis, and

functional expression of a novel growth regulator, oncostatin M. *Mol. Cell. Biol.* **9** 2847-2853.

Malle-E, Bollmann-A, Steinmetz-A, Gemsa-D, Leis-HJ, and Sattler-W (1997) Serum amyloid A (SAA) protein enhances formation of cyclooxygenase metabolites of activated human monocytes. *FEBS. Lett.* **419** 215-219.

Mann-KG, Jenny-RJ, and Krishnaswamy-S (1988) Cofactor proteins in the assembly and expression of blood clotting enzyme complexes. *Annu. Rev. Biochem* **57** 915-956.

Mantzouranis-EC, Dowton-SB, Whitehead-AS, Edge-MD, Bruns-GA, and Colten-HR (1985) Human serum amyloid P component. cDNA isolation, complete sequence of pre-serum amyloid P component, and localization of the gene to chromosome 1. *J. Biol. Chem.* **260** 7752-7756
.

March-CJ, Mosley-B, Larsen-A, Cerretti-DP, Braedt-G, Price-V, Gillis-S, Henney-CS, Kronheim-SR, Grabstein-K, and et-al (1985) Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature.* **315** 641-647.

Marhaug-G, Sletten-K, and Husby-G (1982) Characterisation of amyloid related protein SAA complexed with serum lipoproteins (apoSAA) *Clin. Exp. Immunol.* **50** 382-389.

Masters-CL, Multhaup-G, Simms-G, Pottgiesser-J, Martins-RN, and Beyreuther-K (1985) Neuronal origin of a cerebral amyloid: neurofibrillary tangles of Alzheimer's disease contain the same protein as the amyloid of plaque cores and blood vessels. *EMBO. J.* **4** 2757-2763.

Maxam-AM, and Gilbert-W (1992) A new method for sequencing DNA. 1977 *Biotechnology.* **24** 99-103.

May-LT, Santhanam-U, Tatter-SB, Bhardwaj-N, Ghrayeb-J, and Sehgal-PB (1988) Phosphorylation of secreted forms of human beta 2-interferon/hepatocyte stimulating factor/interleukin-6. *Biochem. Biophys. Res. Commun.* **152** 1144-1150.

McKean-DJ, Huntoon-C, and Bell-M (1994) Ligand-induced desensitization of interleukin 1 receptor-initiated intracellular signaling events in T helper lymphocytes. *J. Exp. Med* **180** 1321-1328.

McKean-DJ, Podzorski-RP, Bell-MP, Nilson-AE, Huntoon-CJ, Slack-J, Dower-SK, and Sims-J (1993) Murine T helper cell-2 lymphocytes express type I and type II IL-1 receptors, but only the type I receptor mediates costimulatory activity. *J. Immunol.* **151** 3500-3510 .

McMahan-CJ, Slack-JL, Mosley-B, Cosman-D, Lupton-SD, Brunton-LL, Grubin-CE, Wignall-JM, Jenkins-NA, Brannan-CI, and et-al (1991) A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types. *EMBO J* **10** 2821-2832 .

Meek-RL, and Benditt-EP (1989) Rat tissues express serum amyloid A-related mRNAs. *Proc. Nat. Acad. Sci.* **86** 1890-1894.

Meek-RL, Eriksen-N, and Benditt-EP (1992) Murine serum amyloid A3 is a high density apolipoprotein and is secreted by macrophages. *Proc. Natl. Acad. Sci. U. S. A.* **89** 7949-7952.

Meek-RL, Hoffman-JS, and Benditt-EP (1986) Amyloidogenesis. One serum amyloid A isotype is selectively removed from the circulation. *J. Exp. Med* **163** 499-510.

Meek-RL, Urieli-Shoval-S, and Benditt-EP (1994) Expression of apolipoprotein serum amyloid A mRNA in human atherosclerotic lesions and cultured vascular cells: implications for serum amyloid A function. *Proc. Natl. Acad. Sci. U. S. A.* **91** 3186-3190.

Mendel-DB, Khavari-PA, Conley-PB, Graves-MK, Hansen-LP, Admon-A, and Crabtree-GR (1991) Characterization of a cofactor that regulates dimerization of a mammalian homeodomain protein. *Science.* **254** 1762-1767 .

Metcalf-D, Waring-P, and Nicola-NA (1992) Actions of leukaemia inhibitory factor on megakaryocyte and platelet formation. *Ciba. Found. Symp.* **167** 174-87.

Miagkov-AV, Kovalenko-DV, Brown-CE, Didsbury-JR, Cogswell-JP, Stimpson-SA, Baldwin-AS, and Makarov-SS (1998) NF-kappaB activation provides the potential link between inflammation and hyperplasia in the arthritic joint. *Proc. Natl. Acad. Sci. U. S. A* **95** 13859-13864 .

Mietus-Snyder-M, Sladek-FM, Ginsburg-GS, Kuo-CF, Ladias-JAA, Darnell-JE Jr, and Karathanasis-SK (1992) Antagonism between apolipoprotein AI regulatory protein 1, Ear3/COUP-TF, and hepatocyte nuclear factor 4 modulates apolipoprotein CIII gene expression in liver and intestinal cells *Mol. Cell. Biol.* **12** 1708-1718.

Mitchell-TI, Coon-CI, and 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.

Moody-BJ (1982) Changes in the serum concentrations of thyroxine-binding prealbumin and retinol-binding protein following burn injury. *Clin. Chim. Acta.* **118** 87-92.

Moshage-H (1997) Cytokines and the hepatic acute phase response. *J. Pathol.* **181** 257-266.

Munoz-E, Beutner-U, Zubiaga-A, and Huber-BT (1990) IL-1 activates two separate signal transduction pathways in T helper type II cells. *J.Immunol.* **144** 964-969 .

Nakajima-T, Kinoshita-S, Sasagawa-T, Sasaki-K, Naruto-M, Kishimoto-T, and Akira-S (1993) Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. *Proc-Natl-Acad-Sci-U-S-A.* **90** 2207-2211.

Natesan-S, and Gilman-MZ (1993) DNA bending and orientation-dependent function of YY1 in the c-fos promoter. *Genes Dev.* **7** 2497-2509 .

Nelson-SR, Lyon-M, Gallagher-JT, Johnson-EA, and Pepys-MB (1991) Isolation and characterization of the integral glycosaminoglycan constituents of human amyloid A and monoclonal light-chain amyloid fibrils. *Biochem. J.* **275** 67-73 .

Neri-A, Chang-CC, Lombardi-L, Salina-M, Corradini-P, Maiolo-AT, Chaganti-RS, and Dalla-Favera-R (1991) B cell lymphoma-associated chromosomal translocation involves candidate oncogene lyt-10, homologous to NF-kappa B p50. *Cell.* **67** 1075-1087 .

Nicola-NA (1994) Cytokines and their receptors Nicola-NA (ed) Oxford University Press, Oxford

Nolan-GP, Ghosh-S, Liou-HC, Tempst-P, and Baltimore-D (1991) DNA binding and I kappa B inhibition of the cloned p65 subunit of NF-kappa B, a rel-related polypeptide. *Cell.* **64** 961-969.

Osborn-L, Kunkel-S, and Nabel-GJ (1989) Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proc. Natl. Acad. Sci. U. S. A.* **86** 2336-2340 .

Parks-JS, and Rudel-LL (1985) Alteration of high density lipoprotein subfraction distribution with induction of serum amyloid A protein (SAA) in the nonhuman primate. *J. Lipid. Res.* **26** 82-91.

Parmelee-DC, Titani-K, Ericsson-LH, Eriksen-N, Benditt-EP, and Walsh-KA (1982) Amino acid sequence of amyloid-related apoprotein (apoSAA1) from human high-density lipoprotein. *Biochemistry.* **21** 3298-3303.

Patel-H, Bramall-J, Waters-H, De-Beer-MC, and Woo-P (1996) Expression of recombinant human serum amyloid A in mammalian cells and demonstration of the region necessary for high-density lipoprotein binding and amyloid fibril formation by site-directed mutagenesis. *Biochem. J.* **318** 1041-1049.

Paul-SR, Bennett-F, Calvetti-JA, Kelleher-K, Wood-CR, O'Hara-RM Jr, Leary-AC, Sibley-B, Clark-SC, Williams-DA, and et-al (1990) Molecular cloning of a cDNA encoding interleukin 11, a stromal cell-derived lymphopoietic and hematopoietic cytokine. *Proc. Natl. Acad. Sci. U. S. A.* **87** 7512-7516.

Pincus-T, and Callahan-LF (1986) Taking Mortality in Rheumatoid Arthritis Seriously - Predictive Markers, Socioeconomic Status and Comorbidity *The Journal of Rheumatology* **13** 841-845.

Preciado-Patt-L, Pras-M, and Fridkin-M (1996a) Binding of human serum amyloid A (hSAA) and its high-density lipoprotein3 complex (hSAA-HDL3) to human neutrophils. Possible implication to the function of a protein of an unknown physiological role. *Int. J. Pept. Protein. Res.* **48** 503-513.

Preciado-Patt-L, Hershkoviz-R, Fridkin-M, and Lider-O (1996b) Serum amyloid A binds specific extracellular matrix glycoproteins and induces the adhesion of resting CD4+ T cells. *J. Immunol.* **156** 1189-1195.

Proud-D, and Kaplan-AP (1988) Kinin formation: mechanisms and role in inflammatory

disorders. *Annu. Rev. Immunol.* **6** 49-83.

Pruzanski-W, de-Beer-FC, de-Beer-MC, Stefanski-E, and Vadas-P (1995) Serum amyloid A protein enhances the activity of secretory non-pancreatic phospholipase A2. *Biochem. J.* **309** 461-464.

Puren-AJ, Fantuzzi-G, Gu-Y, Su-MS, and Dinarello-CA (1998) Interleukin-18 (IFNgamma-inducing factor) induces IL-8 and IL-1beta via TNFalpha production from non-CD14+ human blood mononuclear cells. *J. Clin. Invest.* **101** 711-721.

Quesniaux-VF, Clark-SC, Turner-K, and Fagg-B (1992) Interleukin-11 stimulates multiple phases of erythropoiesis in vitro. *Blood* **80** 1218-23.

Raich-N, Clegg-CH, Grofti-J, Romeo-PH, and Stamatoyannopoulos-G (1995) GATA1 and YY1 are developmental repressors of the human epsilon-globin gene. *EMBO J.* **14** 801-809

.

Ramadori-G, Sipe-JD, Dinarello-CA, Mizel-SB, and Colten-HR (1985) 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.

Raught-B, Khursheed-B, Kazansky-A, and Rosen-J (1994) YY1 represses beta-casein gene expression by preventing the formation of a lactation-associated complex. *Mol. Cell. Biol.* **14** 1752-1763.

Ray-A, Hannink-M, and Ray-BK (1995) Concerted participation of NF-kappa B and C/EBP heteromer in lipopolysaccharide induction of serum amyloid A gene expression in liver. *J. Biol. Chem.* **270** 7365-7374.

Ray-A, and Ray-BK (1996) A novel cis-acting element is essential for cytokine mediated transcriptional induction of the serum amyloid A gene in nonhepatic cells *Mol Cell Biol* **16** 1584-1594.

Ray-BK, and Ray-A (1997) Involvement of an SAF-like transcription factor in the activation of serum amyloid A gene in monocyte/macrophage cells by lipopolysaccharide. *Biochemistry.* **36** 4662-8.

Rende-M, Muir-D, Ruoslahti-E, Hagg-T, Varon-S, and Manthorpe-M (1992) Immunolocalization of ciliary neuronotrophic factor in adult rat sciatic nerve. *Glia.* **5** 25-32.

Rey-Campos-J, Chouard-T, Yaniv-M, and Cereghini-S (1991) vHNF1 is a homeoprotein that activates transcription and forms heterodimers with HNF1 *EMBO. J.* **10** 1445-1457.

Richardson-CC (1965) Phosphorylation of nucleic acid by an enzyme from T4 bacteriophage-infected Escherichia coli. *Proc. Natl. Acad. Sci. U. S. A.* **54** 158-165.

Robey-FA, Jones-KD, and Steinberg-AD (1985) C-reactive protein mediates the solubilization of nuclear DNA by complement in vitro. *J. Exp. Med.* **161** 1344-1356.

Roitt-I, Brostoff-J, and Male-D (1996) Inflammation in Immunology Mosby Publ, Turin

Ross-R (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature.* **362** 801-809.

Rosenwasser-LJ, Webb-AC, Clark-BD, Irie-S, Chang-L, Dinarello-CA, Gehrke-L, Wolff-SM, Rich-A, and Auron-PE (1986) Expression of biologically active human interleukin 1 subpeptides by transfected simian COS cells. *Proc. Natl. Acad. Sci. U. S. A.* **83** 5243-5246.

Rubio-N, Sharp-PM, Rits-M, Zahedi-K, and Whitehead-AS (1993) Structure, expression, and evolution of guinea pig serum amyloid P component and C-reactive protein. *J. Biochem. Tokyo.* **113** 277-284 .

Ryseck-RP, Weih-F, Carrasco-D, and Bravo-R (1996) RelB, a member of the Rel/NF-kappa B family of transcription factors. *Braz. J. Med. Biol. Res.* **29** 895-903 .

Saklatvala-J, Bird-TA, Kaur-P, and O'Neill-LA (1990) IL-1 signal transduction: evidence of activation of G protein and protein kinase. *Prog. Clin. Biol. Res.* **349** 285-295 .

Samadani-U, and Costa-RH (1996) The transcriptional activator hepatocyte nuclear factor 6 regulates liver gene expression. *Mol. Cell. Biol.* **16** 6273-6284.

Sanger-F, Nicklen-S, and Coulson-AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U. S. A.* **74** 5463-5467.

Santhanam-U, Ghrayeb-J, Sehgal-PB, and May-LT (1989) Post-translational modifications of human interleukin-6. *Arch. Biochem. Biophys.* **274** 161-170.

Sellar-GC, Oghene-K, Boyle-S, Bickmore-WA, and Whitehead-AS (1994) Organization of the region encompassing the human serum amyloid A (SAA) gene family on chromosome 11p15.1. *Genomics.* **23** 492-495.

Sen-R, and Baltimore-D (1986) Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. *Cell.* **47** 921-928.

Sen-R, and Baltimore-D (1986) Multiple nuclear factors interact with the immunoglobulin

enhancer sequences. *Cell.* **46** 705-716 .

Sendtner-M, Stockli-KA, and Thoenen-H (1992) Synthesis and localization of ciliary neurotrophic factor in the sciatic nerve of the adult rat after lesion and during regeneration. *J. Cell. Biol.* **118** 139-148.

Shachter-NS, Zhu-Y, Walsh-A, Breslow-JL, and Smith-JD (1993) Localization of a liver-specific enhancer in the apolipoprotein E/C-I/C-II gene locus. *J. Lipid. Res.* **34** 1699-1707

.

Shephard-EG, Anderson-R, Strachan-AF, Kuhn-SH, and De-Beer-FC (1986) CRP and neutrophils: functional effects and complex uptake. *Clin. Exp. Immunol.* **63** 718-727 .

Shi-Y, Seto-E, Chang-LS, and Shenk-T (1991) Transcriptional repression by YY1, a human GLI-Kruppel-related protein, and relief of repression by adenovirus E1A protein. *Cell* **67** 377-388 .

Shimizu-Y, Newman-W, Gopal-TV, Horgan-KJ, Graber-N, Beall-LD, van-Seventer-GA, and Shaw-S (1991) Four molecular pathways of T cell adhesion to endothelial cells: roles of LFA-1, VCAM-1, and ELAM-1 and changes in pathway hierarchy under different activation conditions. *J. Cell. Biol.* **113** 1203-1212.

Siebenlist-U, Franzoso-G, and Brown-K (1994) Structure, regulation and function of NF-kappa B. *Ann. Rev. Cell. Biol.* **10** 405-455 .

Sims-JE, March-CJ, Cosman-D, Widmer-MB, MacDonald-HR, McMahan-CJ, Grubin-CE, Wignall-JM, Jackson-JL, Call-SM, and et-al (1988) cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. *Science* **241** 585-589 .

Skinner-M, and Cohen-AS (1988) Amyloid P component. *Methods. Enzymol.* **163** 523-536

.

Springer-TA (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell.* **76** 301-314.

Stahl-N, Boulton-TG, Farruggella-T, Ip-NY, Davis-S, Witthuhn-BA, Quelle-FW, Silvennoinen-O, Barbieri-G, and Pellegrini-S (1994) Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. *Science.* **263** 92-95 .

Steel-DM, Donoghue-FC, O'Neill-RM, Uhlar-CM, and Whitehead-AS (1996) Expression and regulation of constitutive and acute phase serum amyloid A mRNAs in hepatic and non-hepatic cell lines. *Scand. J. Immunol.* **44** 493-500.

Stewart-CL, Kaspar-P, Brunet-LJ, Bhatt-H, Gadi-I, Kontgen-F, and Abbondanzo-SJ (1992) Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor *Nature.* **359** 76-79.

Strachan-AF, Brandt-WF, Woo-P, van-der-Westhuyzen-DR, Coetzee-GA, de-Beer-MC, Shephard-EG, and de-Beer-FC (1989) 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.

Strauss-S, Bauer-J, Ganter-U, Jonas-U, Berger-M, and Volk-B (1992) Detection of interleukin-6 and alpha 2-macroglobulin immunoreactivity in cortex and hippocampus of Alzheimer's disease patients. *Lab. Invest.* **66** 223-230 .

Stylianou-E, O'Neill-LA, Rawlinson-L, Edbrooke-MR, Woo-P, and Saklatvala-J (1992)

Interleukin 1 induces NF-kappa B through its type I but not its type II receptor in lymphocytes. *J. Biol. Chem.* **267** 15836-15841.

Taga-T, Hibi-M, Hirata-Y, Yamasaki-K, Yasukawa-K, Matsuda-T, Hirano-T, and Kishimoto-T (1989) Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell.* **58** 573-581 .

Taga-T, Hibi-M, Hirata-Y, Yamasaki-K, Yasukawa-K, Matsuda-T, Hirano-T, and Kishimoto-T (1989) Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell.* **58** 573-581.

Taga-T, and Kishimoto-T (1992) Role of a two-chain IL-6 receptor system in immune and hematopoietic cell regulation. *Crit. Rev. Immunol.* **11** 265-280.

Tape-C, Tan-R, Nesheim-M, and Kisilevsky-R (1988) Direct evidence for circulating apoSAA as the precursor of tissue AA amyloid deposits. *Scand. J. Immunol.* **28** 317-324 .

Tronche-F, and Yaniv-M (1992) HNF1, a homeoprotein member of the hepatic transcription regulatory network. *BioEssays* **14** 579-587 .

Turnell-W, Sarra-R, Glover-ID, Baum-JO, Caspi-D, Baltz-ML, and Pepys-MB (1986) Secondary structure prediction of human SAA1. Presumptive identification of calcium and lipid binding sites. *Mol. Biol. Med.* **3** 387-407.

Urban-MB, Schreck-R, and Baeuerle-PA (1991) NF-kappa B contacts DNA by a heterodimer of the p50 and p65 subunit. *EMBO. J.* **10** 1817-1825 .

Urdal-DL, Call-SM, Jackson-JL, and Dower-SK (1988) Affinity purification and chemical

analysis of the interleukin-1 receptor. *J. Biol. Chem.* **263** 2870-2877.

Urieli-Shoval-S, Meek-RL, Hanson-RH, Eriksen-N, and Benditt-EP (1994) Human serum amyloid A genes are expressed in monocyte/macrophage cell lines. *Am. J. Pathol.* **145** 650-660.

VanLenten-BJ, Hama-SY, deBeer-FC, Stafforni-DM, McIntyre-TM, Prescott-SM, LaDu-BN, Fogelman-AM, and Navab-M (1995) Anti-inflammatory HDL becomes pro-inflammatory during the acute phase response *J. Clin. Invest* **96** 2758-2767.

Verbeek-MM, Otte-Holler-I, Westphal-JR, Wesseling-P, Ruiter-DJ, and de-Waal-RM (1994) Accumulation of intercellular adhesion molecule-1 in senile plaques in brain tissue of patients with Alzheimer's disease. *Am. J. Pathol.* **144** 104-116.

Vincent-CK, Gualberto-A, Patel-CV, and Walsh-K (1993) Different regulatory sequences control creatine kinase-M gene expression in directly injected skeletal and cardiac muscle. *Mol. Cell. Biol.* **13** 1264-1272.

Volanakis-JE, and Kaplan-MH (1974) Interaction of C-reactive protein complexes with the complement system. II. Consumption of guinea pig complement by CRP complexes: requirement for human C1q. *J. Immunol.* **113** 9-17.

Watson-G, Coade-S, and Woo-P (1992) Analysis of the genomic and derived protein structure of a novel human serum amyloid A gene, SAA4. *Scand. J. Immunol.* **36** 703-712.

Whitehead-AS, Bruns-GA, Markham-AF, Colten-HR, and Woods-DE (1983) Isolation of human C-reactive protein complementary DNA and localization of the gene to chromosome 1.

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Science. **221** 69-71 .

Whitehead-AS, de-Beer-MC, Steel-DM, Rits-M, Lelias-JM, Lane-WS, and de-Beer-FC (1992) Identification of novel members of the serum amyloid A protein superfamily as constitutive apolipoproteins of high density lipoprotein. *J. Biol. Chem.* **267** 3862-3867.

Wigler-M, Silverstein-S, Lee-LS, Pellicer-A, Cheng-Yc, and Axel-R (1977) Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. *Cell* **11** 223-232 .

Williams-RL, Hilton-DJ, Pease-S, Willson-TA, Stewart-CL, Gearing-DP, Wagner-EF, Metcalf-D, Nicola-NA, and Gough-NM (1988) Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature.* **336** 684-687 .

Wood-DD, Ihrie-EJ, Dinarello-CA, and Cohen-PL (1983) Isolation of an interleukin-1-like factor from human joint effusions. *Arthritis.Rheum.* **26** 975-983 .

Xanthopoulos-KG, Mirkovitch-J, Decker-T, Kuo-CF, and Darnell-JE Jr (1989) Cell-specific transcriptional control of the mouse DNA-binding protein mC/EBP. *Proc. Natl. Acad. Sci. U. S. A.* **86** 4117-4121 .

Xia-C, Cheshire-JK, Patel-H, and Woo-P (1997) Cross-talk between transcription factors NF-kappa B and C/EBP in the transcriptional regulation of genes. *Int. J. Biochem. Cell. Biol.* **29** 1525-1539.

Xu-L, Badolato-R, Murphy-WJ, Longo-DL, Anver-M, Hale-S, Oppenheim-JJ, and Wang-JM (1995) A Novel Biologic Function of Serum Amyloid A: Induction of T Lymphocyte Migration and Adhesion *The Journal of Immunology* **155** 1184-1190.

Yamamoto-K, Goto-N, Kosaka-J, Shiroo-M, Yeul-YD, and Migita-S (1987) Structural diversity of murine serum amyloid A genes. Evolutionary implications. *J. Immunol.* **139** 1683-1688.

Yamasaki-K, Taga-T, Hirata-Y, Yawata-H, Kawanishi-Y, Seed-B, Taniguchi-T, Hirano-T, and Kishimoto-T (1988) Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor. *Science.* **241** 825-828 .

Yoshizaki-K, Matsuda-T, Nishimoto-N, Kuritani-T, Taeho-L, Aozasa-K, Nakahata-T, Kawai-H, Tagoh-H, Komori-T, and et-al (1989) Pathogenic significance of interleukin-6 (IL-6/BSF-2) in Castleman's disease. *Blood.* **74** 1360-1367.

Zarling-JM, Shoyab-M, Marquardt-H, Hanson-MB, Lioubin-MN, and Todaro-GJ (1986) Oncostatin M: a growth regulator produced by differentiated histiocytic lymphoma cells. *Proc-Natl-Acad-Sci-U-S-A.* **83** 9739-9743.

Zhang-YH, Lin-JX, Yip-YK, and Vilcek-J (1988) Enhancement of cAMP levels and of protein kinase activity by tumor necrosis factor and interleukin 1 in human fibroblasts: role in the induction of interleukin 6. *Proc. Natl. Acad. Sci. U. S. A* **85** 6802-6805 .

Zhou-Q, and Engel-DA (1995) Adenovirus E1A243 disrupts the ATF/CREB-YY1 complex at the mouse c-fos promoter. *J. Virol.* **69** 7402-7409 .