Oncostatin M induced α_1-antitrypsin (AAT) gene expression in Hep G2 cells is mediated by a 3′ enhancer

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α_1-Antitrypsin (AAT) is the major serine proteinase inhibitor (SERPIN A1) in human plasma. Its target proteinase is neutrophil elastase and its main physiological function is protection of the lower respiratory tract from the destructive effects of neutrophil elastase during an inflammatory response. Circulating levels of AAT rise 2–3-fold during inflammation and the liver produces most of this increase. The cytokines oncostatin M (OSM) and interleukin-6 have been shown to be mainly responsible for this effect, which is mediated via the interaction of cytokine-inducible transcription factors with regulatory elements within the gene. In the present study, we report for the first time that OSM stimulation of hepatocyte AAT occurs via an interaction between the hepatocyte promoter and an OSM-responsive element at the 3′-end of the AAT gene. This effect is mediated by the transcription factor signal transducer and activator of transcription 3 ('STAT 3') binding to an OSM-responsive element (sequence TTCTCTTAA), and this site is distinct from, but close to, a previously reported interleukin-6-responsive element.

Key words: acute-phase response, cytokines, gene regulation, liver cells, SERPIN A1.

INTRODUCTION

α_1-Antitrypsin (AAT), also known as α_1-proteinase inhibitor ('α_1 PI'), is the major serine proteinase inhibitor (serpin) in human plasma [1], and its principal site of synthesis is in the liver [2]. Although AAT has broad specificity, its main target proteinase is neutrophil elastase released by neutrophils during inflammation [3]. Its physiological role is to protect the lower respiratory tract from the damaging effects of uninhibited neutrophil elastase [4]. Other cell types, including monocytes/macrophages [5], bronchial epithelial cells and lung alveolar epithelial cells [6,7], make AAT to lesser amounts. Local production may be an important mechanism for controlling serine proteinase activity at sites of tissue injury [8,9].

AAT gene expression in the liver increases 2–3-fold during inflammation and this increase is referred to as the acute-phase response. In general, acute-phase response genes can be divided into two groups. The cytokine interleukin (IL)-1, or combinations of IL-1 and IL-6 [10] regulate type I genes, whereas type II genes, of which AAT is a member, respond mainly to the IL-6-like cytokines, including oncostatin M (OSM) [11]. These two cytokines, IL-6 and OSM, are the major stimuli for the AAT acute-phase response. They mediate their effects via the gp130 signal transducer molecule, whose role is to initiate a cascade of events resulting in the phosphorylation of transcription factors, thereby activating them and permitting their binding to DNA. Phosphorylation occurs principally at tyrosine residues for the signal transducers and activators of transcription (STAT) factors [12,13] and at threonine residues for nuclear factor (NF)-IL-6 [14]. Two types of DNA sequence mediate the IL-6 response. Type I elements bind members of the CAAT-enhancer-binding protein (C/EBP) family of transcription factors, including NF-IL-6 [15], whereas type II elements (also known as acute-phase response elements) are involved in the Janus kinase ('JAK')/STAT pathway [16]. Our previous studies [17] have mapped DNA sequence elements in the 3′ enhancer region of the AAT gene through which IL-6 mediates its effect via the C/EBP family of transcription factors. These studies also demonstrated the requirement for 5′ regulatory elements to detect maximal IL-6 responses in the liver hepatoma cell line Hep G2. In monocytes/macrophages, lipopolysaccharide (LPS) is capable of up-regulating the AAT gene [18], whereas no effect was seen in hepatocytes (P. Masters, K. Morgan and N. Kalsheker, unpublished work). It has been shown that the LPS increase in AAT gene expression is mediated via an IL-1-type NF-κB effect [19]. It has also been demonstrated that lung alveolar epithelial cells (A549) are also capable of up-regulating AAT synthesis in response to OSM [6]. AAT protein output is stimulated up to 3-fold by both IL-6 and OSM in Hep G2 cells [6] and, quantitatively, this accounts for the bulk of circulating AAT. Collectively, all of this data infers that at least three pathways exist for the up-regulation of the AAT gene, which are dependent on the stimulus, i.e. IL-6, OSM or LPS.

The AAT gene consists of seven exons: 1A, 1B and 1C together with exons 2–5. Exons 2–5 code for the protein and exons 1A, 1B and 1C are differentially spliced. There are distinct promoters for hepatocytes and monocyte/macrophages [20]. There are three possible monocyte transcriptional start sites designated M1, M2 and M3, which generate transcripts of 2.0, 1.95 and 1.8 kb respectively [20], in addition to the 1.6 kb hepatocyte

Abbreviations used: AP-1, activating protein 1; AAT, α_1-antitrypsin; C/EBP, CAAT-enhancer-binding protein; IL, interleukin; LIF, leukaemia inhibitory factor; LPS, lipopolysaccharide; OSM, oncostatin M; ORE, OSM-responsive element; NF, nuclear factor; STAT, signal transducer and activator of transcription; SV40, simian virus 40.

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transcript [21]. A distinct corneal start site, which also generates a 1.6 kb transcript, has been documented [22]. Transcripts emanating from each of the transcriptional start sites listed above account for five of the species, and the sixth is generated by excision of exon 1B from the full-length M1 monocyte transcript [20], resulting in a 1.8 kb transcript. The biological significance of these alternative transcripts has yet to be established, but they may differ in terms of RNA stability and/or transitional efficiency. Stimulation of AAT in the leukaemic monocyte cell line (U937) by IL-6 has been shown previously to preferentially remove exon 1B from AAT mRNA [23], suggesting that this transcript may play a role in AAT gene expression. Traditionally it has been assumed that the hepatocyte promoter and the monocyte/macrophage promoter are tissue specific [20,24]. However, it has been shown that the liver is capable of making monocyte/macrophage transcripts when stimulated with the inflammatory cytokine IL-6 [25], albeit at much lower levels than that generated from the hepatocyte promoter.

In order to gain a better understanding of the mechanisms by which OSM regulates AAT gene expression in the liver, we have identified an OSM-responsive element (ORE) in a 3′ enhancer that binds STAT3.

EXPERIMENTAL

Cytokines

Recombinant human OSM was obtained from R & D Systems (Abingdon, Oxon., U.K.) and was used at a final concentration of 100 ng/ml in complete medium [minimal essential medium with Earle's salts supplemented with 10 % (v/v) heat-inactivated foetal bovine serum, 1 % L-glutamine, 1 % penicillin/streptomycin and 1 % fungizone (Gibco BRL, Paisley, Renfrewshire, Scotland, U.K.)] for stimulation of Hep G2 cells. Recombinant human IL-6 was purchased from Promega (Southampton, U.K.) and was used at a final concentration of 90 ng/ml. Recombinant human leukaemia inhibitory factor (LIF) was obtained from Chemicon International (Harrow, Middx., U.K.) and used at a final concentration of 100 units/ml. Previous work has shown these concentrations to be optimal (P. Masters, K. Morgan and N. Kalsheker, unpublished work).

Oligonucleotides

All oligonucleotides were synthesized in the Biopolymer and Synthesis Analysis Unit, University of Nottingham, Nottingham, U.K., and were end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Helena BioSciences, Sunderland, U.K.).

Cell culture

Hep G2 cells were maintained in culture in complete medium. The cells were incubated at 37 °C in a humidified atmosphere of 5 % CO_2. Cells were allowed to reach at least 80 % confluency prior to subculturing. The cells were washed with PBS and then detached from the surface of the plastic tissue culture flask by trypsinization (Gibco BRL) and inoculated into fresh complete medium at a density of 30 %.

Generation of reporter gene constructs

The two constructs used in this study were prepared using the firefly luciferase reporter gene pGL3P vector [containing a simian virus 40 (SV40) promoter] (Promega). AAT fragments from the 5′-end of the gene (Figure 1a) and 3′-end (Figure 1b) were cloned into pGL3P by using PCR primers, which also incorporated the requisite restriction sites for cloning. The 5′ sequence was cloned into the *Kpn*1/*Xho*1 sites upstream of the luciferase gene and the 3′ sequence into the *Bam*H1/*Sal*1 sites downstream of the luciferase gene. A plasmid, designated pAAT5′/3′, contained the human AAT 5′ (−275 to −35) and the 3′ (+11167 to +11536) flanking sequences. All co-ordinates are relative to the hepatocyte transcriptional start site located at +1, as shown in Figure 1(a). This construct has been used previously [17] to map a hepatocyte IL-6-response element in the 3′ AAT enhancer region. Another plasmid containing the same 5′ sequence together with 134 bp of additional 3′ sequence extending in total from +11167 to +11670, designated pAAT5′/3′+STAT, was also generated, since an element with a perfect match to the STAT consensus was identified (the proposed ORE) in the extra 3′ sequence. All the constructs had their sequence confirmed (ABI Prism 310).

Site-directed mutagenesis

The first two bases of the proposed ORE (TTCTCTTAA) were mutated (GGCTCTTAA; altered bases are underlined), using the GeneEditor *in vitro* site-directed mutagenesis system (Promega), to generate the construct pAAT5′/3′+mutated STAT. This was accomplished by using the mutagenic primer 5′-ACACAAGTCTCCTGGGCTCTTAAGCTTACT-3′ and the pAAT5′/3′+STAT construct as a template. The successful introduction of the changes was verified by sequencing.

Transfection

In all cases, transfections were performed in triplicate. Plates were set up with wells containing pGL3C (positive control, containing the SV40 promoter and enhancer) or pGL3P (containing the SV40 promoter alone), together with the test constructs pAAT5′/3′, pAAT5′/3′+STAT and pAAT5′/3′+mutated STAT. All wells were co-transfected with a *Renilla* luciferase plasmid (pRL) in order to control for transfection efficiency. All plasmid used in transfection experiments were endotoxin-free preparations (Qiagen, Crawley, West Sussex, U.K.).

Cells were trypsinized, seeded into 12-well plates and grown to 50–80 % confluency prior to transfection. Plasmid DNAs were diluted in 400 μl of serum-free medium (complete medium without serum) per well in order to give a final concentration of 200 ng of luciferase construct and 20 ng of pRL/well. Tfx-50 (Promega) reagent was used at a charge ratio of 3:1 to allow entry of DNA into cells. Tfx-50 reagent was added to the DNA/medium mixture and vortexed immediately. This was then incubated at 22 °C for 15 min to allow DNA–Tfx-50 complex formation. Cells were prepared for transfection by removal of the complete medium and washing with PBS. The transfection mix (400 μl/well) was then added to the cells. The plates were incubated at 37 °C for 1 h with an additional 800 μl of complete medium to give a final volume of 1.2 ml/well. The plates were then incubated at 37 °C for a further 24 h prior to cytokine stimulation. Following the 24 h post-transfection incubation period, the complete medium was removed and the cells were washed with PBS. The cells were then covered with 1.2 ml of serum-free medium in the absence (to detect basal levels) or presence of 100 ng/ml OSM, 90 ng/ml IL-6, both OSM and IL-6 at these concentrations or 100 units/ml LIF, as appropriate. Cells were then incubated at 37 °C for a further 24 h, prior to washing and treatment with passive lysis buffer (Promega). The complete medium was removed and the cells were

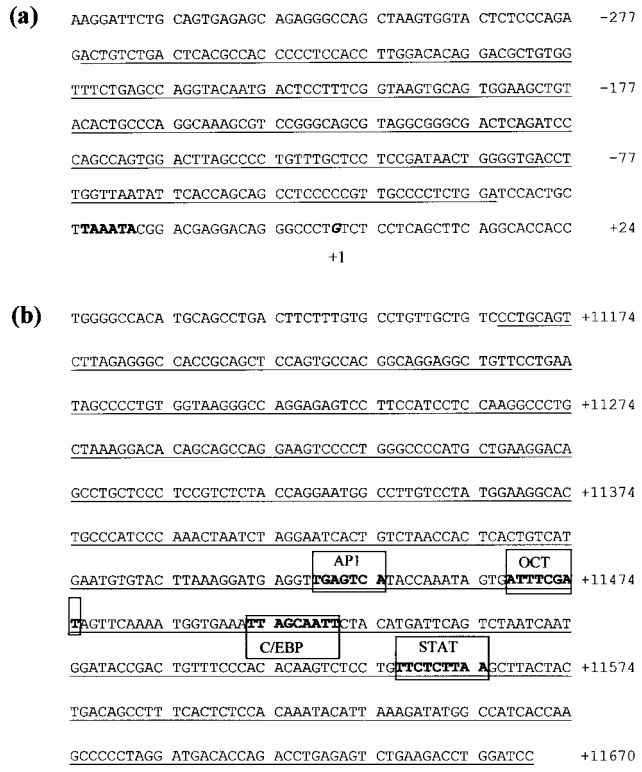

Figure 1 Sequence of the AAT fragments used in the luciferase reporter gene construct

(**a**) The 5′ insert (−275 to −35) is underlined, the TATA box is in bold, and the hepatocyte transcriptional start site is shown in bold and italics at position +1. This sequence includes previously reported tissue-specific (−142 to −42) and enhancer (−266 to −215) regions [31–33]. (**b**) The 3′ insert is underlined. The insert without the STAT site is taken from nucleotide position +11167 to +11536, whereas the STAT insert incorporates nucleotides from +11167 to +11670. NF-binding sites are depicted in bold and boxed. AP1, AP-1; OCT, octomer-1 transcription factor.

washed with PBS. The PBS was removed completely and 200 μl of passive lysis buffer was added to each well. The plates were incubated at 22 °C for 15 min, and the cell lysates were collected and assayed immediately.

Dual luciferase reporter assays

Luciferase Assay Reagent II and Stop and Glo Reagent (Promega) were prepared according to the manufacturer's instructions. Dual-Luciferase® Reporter assays (Promega) were performed using a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, U.S.A.), allowing sequential readings of firefly and *Renilla* luciferase reporter activities. The luminometer was programmed to allow a 2 s delay period prior to quantification of light output over 10 s for both firefly and *Renilla* luciferases. The ratio of firefly/*Renilla* luciferase activities represent the normalized firefly luciferase activity corrected for transfection efficiency.

Preparation of nuclear extracts

Hep G2 cells were stimulated with OSM and IL-6 for the indicated time periods up to 24 h and nuclear extracts were prepared as described by Andrews and Faller [26] with the modifications of Botelho et al. [27]. All nuclear extracts were snap-frozen and stored in aliquots at −80 °C.

Gel-shift assay

Nuclear extract (2 μl) was incubated with 1 μg of poly(dI-dC) in binding buffer [50 mM Tris/HCl (pH 7.5), 50 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol, 1 mM spermidine and 5% (v/v) glycerol] for 15 min at 4 °C. A total of 40 fmol of ^{32}P-labelled probe was then added, and the binding reaction (12.5 μl) incubated at 4 °C for 60 min. Excess unlabelled oligonucleotide (50-fold) was added to the reaction for competition assays. Samples (3 μl) were separated by electrophoresis on 4% (w/v) polyacrylamide gels in prechilled 0.25 × Tris-borate/EDTA [where 1 × Tris-borate/EDTA is 89 mM Tris/borate and 2 mM EDTA (pH 8.0)] at 20 mA constant current for 25 min and dried prior to autoradiography. Antibodies against STAT1 and STAT3 for factor identification were kindly provided by Professor Peter Shaw, School of Biomedical Sciences, Queen's Medical Centre, Nottingham, U.K. In some circumstances these antibodies appear to prevent binding of the STAT1/STAT3 complexes to DNA without producing a supershift [28]. They were added prior to the addition of radiolabelled probe to the binding reaction and incubated for 1 h at 4 °C. The sequence of the oligonucleotide probe across the putative 3′ AAT ORE was 5′-TCCTG-TTCTCTTAAGCTTA-3′ (STAT consensus sequence is underlined); this was annealed to its complement by heating to 100 °C for 5 min in a solution containing 100 mM $MgCl_2$ and 400 mM Tris/HCl (pH 8.0), followed by cooling to 20 °C.

Statistical analysis

All results are means ± S.E.M. The luciferase reporter gene activities in transfected Hep G2 cells under various conditions were compared using a two-tailed Student's t test. The level for significance was taken as $P < 0.05$.

RESULTS

ORE in the 3′ AAT enhancer

Two constructs were used to map the ORE; one with sequence containing the putative STAT element (bases +11167 to +11670) and the other without it (bases +11167 to +11536), as described in the Experimental section. In order for the full response to be seen, the presence of the 5′ flanking sequence (−275 to −35) in these constructs was essential, as has been reported previously [17].

These two constructs were assayed for functional activity in transfected Hep G2 cells under basal conditions and when stimulated with OSM and/or IL-6. The results are shown in Figure 2 as the fold increases over the pGL3P SV40 promoter plasmid alone. The basal values for promoter activity obtained with pAAT5′/3′ and pAAT5′/3′ + STAT were very similar (6.5 ± 1.2- and 6.2 ± 0.8-fold). After stimulation with IL-6, both constructs resulted in similar increases in reporter gene activity (16.6 ± 2.7 and 17.3 ± 2.6) and was consistent with data obtained previously [17]. However, the inclusion of the extra sequence containing the putative STAT site only had a significant positive effect on OSM responsiveness. Whereas the pAAT5′/3′ construct demonstrated some residual OSM effect (13.5 ± 0.7, $P = 0.03$), this was increased with the pAAT5′/3′ + STAT construct (19.3 ± 1.8, $P = 0.0004$), inferring that the additional sequence contains OSM-responsive activity. When both cytokines were used in combination, the effect was even more dramatic. The pAAT5′/3′

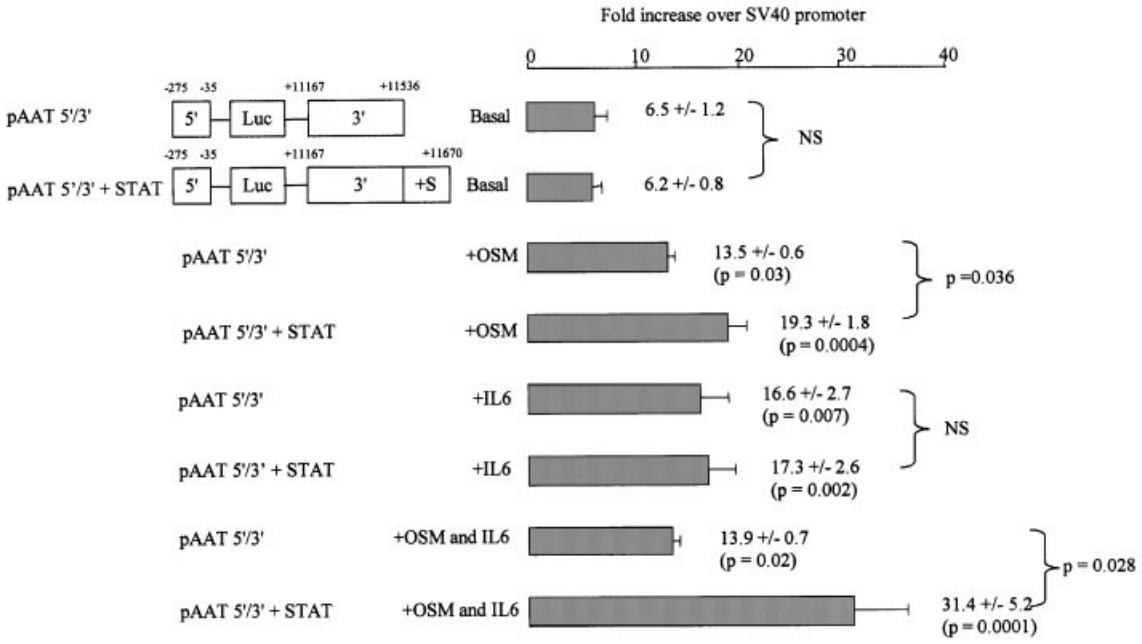

Figure 2 Functional activity of the luciferase reporter gene constructs both with and without the additional putative STAT element in Hep G2 cells

Hep G2 cells were transfected with the reporter gene constructs either with or without the putative STAT (+S) element. Cells were then stimulated for 24 h with OSM and/or IL-6 and luciferase activity was determined. Following stimulation, all the constructs gave significantly higher activities than that seen under basal conditions. Although the IL-6 response remained the same with each of the constructs (with or without the putative STAT element), the OSM response was significantly increased with the pAAT5′3′+STAT construct both on its own ($P = 0.036$) and when used in combination with IL-6 ($P = 0.028$). The basal measurements were from eight sets of experiments and the cytokine stimulations from three experiments, each assayed in triplicate. Results are means ± S.E.M. of the fold increases over the pGL3 SV40 promoter plasmid alone (given the nominal value of 1). NS, not significant.

construct demonstrated a 13.9 ± 0.7-fold increase ($P = 0.02$), similar to the values obtained with each cytokine alone, and this increased to 31.4 ± 5.2-fold ($P = 0.0001$) with pAAT5′/3′+STAT. These data demonstrate that there is a significant additional effect ($P = 0.028$) of OSM in the presence of IL-6 with the construct containing the putative STAT sequence. The 3′ construct alone, either with or without the additional STAT sequence, failed to demonstrate any functional activity under basal and cytokine stimulated conditions (results not shown), highlighting the importance of having the 5′ promoter region in the constructs.

AAT 3′ enhancer ORE binds STAT3

Gel-shift assays were performed to identify the factors binding to the additional 3′ AAT enhancer sequence that was present in the reporter gene construct (pAAT5′/3′+STAT; Figure 2). Since this additional sequence contained a perfect match (TTCTCTTAA) with the STAT consensus (KTMNNNKAA) and stimulation of Hep G2 cells with OSM is known to induce STAT3 production [27], this factor was the most likely candidate. Using the STAT3 consensus sequence (GATCC-TTCTGGGAATTCCTAGATC) as a probe, the presence of STAT3 protein in Hep G2 cells, stimulated for 24 h with OSM, was verified using a STAT3-specific antibody (results not shown). A series of gel shifts were performed using ORE as the probe. Protein binding to the ORE was greatly diminished in the presence of the STAT3 antibody, whereas the STAT1 antibody had no effect (Figure 3a). The binding to the ORE was also eliminated using a 50-fold molar excess of the STAT3 consensus sequence as a competitor. The gel-shift experiments, using ORE as the probe, were repeated with nuclear extracts prepared from Hep G2 cells treated with IL-6 or OSM for various time points from 5 min to 24 h. No STAT3 signal was detected under basal conditions, whereas a strong STAT3 signal was observed upon stimulation with both OSM (Figure 3b) and IL-6 (Figure 3c). The pattern of STAT3 binding to the ORE was very similar for both cytokines, and the signal persisted throughout the time course. Prolonged exposure of these autoradiographs demonstrated the presence of a minor quantity of STAT1 (< 10%), as shown by the presence of STAT1/3 heterodimers and STAT1/1 homodimers (results not shown). Following stimulation with IL-6, a strong band was seen with the C/EBP consensus, which binds IL-6-inducible transcription factors as reported previously [17].

Mutagenesis of the STAT site diminishes the OSM response

The precise location of the functional element mediating the OSM effect was mapped using the construct pAAT5′/3′+mutated STAT, which contained a double mutation introduced at the first two bases of the proposed STAT element. Another series of experiments were performed in which the reporter gene activity of pAAT5′/3′+mutated STAT was compared with pAAT5′/3′+STAT following stimulation for 24 h with OSM, IL-6 and LIF (Table 1). OSM treatment produced a 3.6 ± 0.25-fold increase over basal with the STAT construct, whereas a 1.8 ± 0.45-fold increase was seen with the mutated STAT vector. Mutation of the proposed STAT site significantly ($P = 0.047$) reduced the OSM response to the levels observed with the pAAT5′/3′ construct (2.1 ± 0.1-fold compared with basal; Figure 2). The mutated STAT site had no demonstrable effect on the IL-6 or LIF responses.

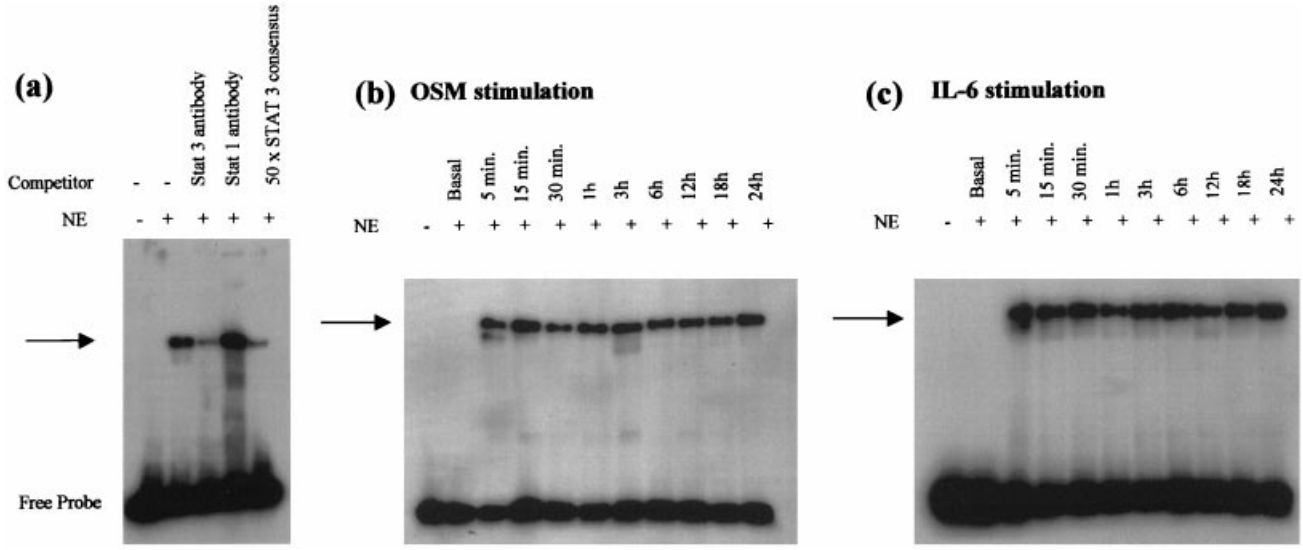

Figure 3 Gel-shift assays

(**a**) Radiolabelled ORE probe was incubated in the absence (−) or presence (+) of nuclear extracts (NE) from Hep G2 cells treated for 24 h with OSM. Extracts were incubated in the absence (−) or presence of STAT1 or STAT3 antibodies or a 50-fold molar excess of the competing STAT3 consensus sequence as indicated. (**b**) Radiolabelled ORE was incubated in the absence (−) or presence (+) of nuclear extracts (NE) prepared from Hep G2 cells under basal conditions or following stimulation with OSM for the indicated times. (**c**) Reactions were as in (**b**), except the cells were treated with IL-6 for the indicated times. Free probe is shown at the bottom of the gels. The arrows indicate the presence of the STAT3–ORE complex.

Table 1 Effect of mutating the proposed STAT site on the OSM, IL-6 and LIF response of the pAAT5′/3′ + STAT luciferase reporter gene construct in Hep G2 cells

Hep G2 cells were transfected with the reporter gene constructs containing either the wild-type or mutated STAT element. Cells were then stimulated for 24 h with OSM, IL-6 or LIF and luciferase activity was determined. From the results in Figure 2, the pAAT5′/3′ + STAT construct has been shown to induce a 3.1-fold (OSM) and 2.8-fold (IL-6) increase over basal levels. LIF was found to produce a 1.4-fold effect over basal. Site-directed mutagenesis of the STAT site resulted in a significant decrease in the OSM response (*$P = 0.047$). This level equated to that seen with the construct that did not contain the extra sequence harbouring the STAT element (2.1-fold increase over basal following treatment with OSM in pAAT5′/3′-transfected cells shown in Figure 2). The IL-6 and LIF effects were not significantly altered by the mutation at the STAT site. Results are means ± S.E.M. of the fold increases over basal activity (in the absence of cytokines) from three sets of experiments each performed in triplicate.

	Cytokine stimulation (fold increase over basal)		
Construct	OSM	IL-6	LIF
pAAT5′/3′ + STAT	3.6 ± 0.25	2.5 ± 0.23	1.4 ± 0.09
pAAT5′/3′ + mutated STAT	1.8 ± 0.45*	3.2 ± 0.64	1.6 ± 0.32

DISCUSSION

In the present study, we have mapped a functional ORE to the 3′-end of the AAT gene, close to a region identified previously as having IL-6-responsive enhancer activity [17], and demonstrated that the ORE is capable of binding the OSM-inducible transcription factor STAT3.

The inclusion of the ORE significantly increased the OSM response and had an additive effect when used in combination with IL-6. Mutagenesis of the proposed STAT3-binding site resulted in a significant decrease of the OSM response, similar to that observed in the absence of the STAT site. There was a small OSM response seen with the construct that did not contain the STAT site. One possible explanation for this may the existence of an activating protein 1 (AP-1) site (which can bind c-Fos/Jun) in the 3′ AAT enhancer located 40 bases upstream of the C/EBP site, which is present in this construct. This site could contribute to the AAT OSM response, as it has been demonstrated that OSM can stimulate c-Fos binding to a transcriptionally responsive AP-1 element in the promoter of the tissue inhibitor of metalloproteinase-1 ('TIMP-1') gene in Hep G2 cells [27]. More recently, it has been shown that there is a co-ordinated interaction between STAT3 and c-Jun in the α_2-macroglobulin gene promoter in OSM-stimulated Hep G2 cells [29]. It is possible that this area of the AAT gene (i.e. 3′ enhancer) is involved in multiple interactions between transcription factors.

The IL-6 effect seen with the constructs both with and without the STAT site was similar (2.8- and 2.6-fold increases over basal respectively), indicating that the ORE was not contributing to the IL-6 response. We have identified previously an NF-IL-6 site within the 3′ AAT enhancer located 55 bases upstream of the STAT site (Figure 1), which mediates the IL-6 response [17], and this site was present in both constructs. This earlier observation is reinforced by the mutagenesis data, which showed that the STAT site was important for the OSM response, but had no effect on the IL-6 response. This infers that the OSM response via the ORE is distinct from the IL-6 pathway in Hep G2 cells. As was noted previously for the IL-6 effect [17], the 5′ AAT flanking sequence from −275 to −35 was essential in order to observe the OSM response. This indicates that 'cross-talk' is likely between the 5′ and 3′ regions, which is needed in order to obtain elevated AAT gene expression.

LIF treatment has been shown previously to result in small increases (approx. 10%) in AAT protein expression in Hep G2 cells [30]. Our reporter gene assays also show that LIF has a small functional effect after 24 h stimulation (1.4-fold over basal) and that mutation of the proposed STAT site had no effect on this response (1.6-fold). As with IL-6, it appears that LIF does not use the newly identified ORE.

The gel-shift experiments clearly demonstrate that the putative STAT site present in the 3′ AAT enhancer region is indeed capable of binding the transcription factor STAT3, as would be predicted, since this site is a perfect match for the STAT consensus. OSM- and IL-6-stimulated Hep G2 cell nuclear extracts contain significant levels of STAT3, which bind to the ORE in both instances over the time course studied and remains strong throughout the 24 h time course. The mutagenesis data demonstrate that, despite binding being seen with both cytokines, only OSM induces a functional response at 24 h via STAT3 binding to the ORE. The molecular mechanism responsible for this difference is not understood at present. An interesting possibility is that other, as yet unidentified, interacting co-activators are involved, which are recruited/tethered by STAT3 and are differentially produced following cytokine stimulation.

A previous study [6] has demonstrated that OSM and IL-6 up-regulate the production of AAT protein in Hep G2 cells by 2- to 3-fold. The mean fold increase in reporter gene activity with the pAAT5′/3′+STAT construct in response to OSM treatment (3.1-fold over basal; Figure 2) is in close agreement with the fold increases seen for AAT protein (2.3-fold) and total AAT mRNA (2.8-fold) in OSM-stimulated Hep G2 cells (results not shown).

Our data suggest that, in Hep G2 cells, the AAT gene is regulated predominantly at the transcriptional level, with post-translational regulation most likely playing a minor role, and this is mediated principally via the hepatocyte promoter. The ORE in the 3′ AAT enhancer mediates the OSM response, and this observation reinforces the modular concept of eukaryotic enhancers. The mapping of this element contributes to our understanding of one of the mechanisms by which the AAT gene is able to respond to cytokines as part of the acute-phase response in hepatocytes.

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