Nuclear factors interacting with an interleukin-6 responsive element of rat α_2-macroglobulin gene

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ABSTRACT

During acute inflammation, a group of liver-derived plasma proteins, acute phase proteins (APPs), increase in concentration. Interleukin-6 (IL-6) is responsible for this increase via the induction of APP gene expression. We have identified an IL-6 responsive *cis*-acting element (IL-6RE) of gene encoding a typical APP, rat α_2-macroglobulin (α_2M). The IL-6RE contains a sequence that is conserved among the 5'-flanking regions of various APP genes. Introduction of mutations into the conserved sequence revealed that the sequence, termed IL-6RE core, is a critical and essential component of IL6-RE. Nuclear factors binding to the IL-6RE core were identified in livers of normal and inflamed rats. Mobility shift pattern and DNase I footprinting profile indicated that the factors from normal and inflamed stages recognized the same sequence but were distinct from each other. These results suggested that the regulation of α_2M gene expression may involve mutually exclusive interaction of stage-specific *trans*-acting factors.

INTRODUCTION

Acute phase proteins (APPs) constitute a group of plasma proteins that increase in serum level during acute phase response to tissue injury and microbial infection, and are considered to be participants in host-defense systems (1, 2). A peptide factor, called hepatocyte-stimulating factor (HSF), has been shown to induce APP synthesis in hepatocytes (1, 2). Recently, a multi-functional cytokine, interleukin-6 (IL-6), has been shown to induce expression of various APP genes *in vitro* (in hepatocyte primary cultures and hepatoma cell lines) (3-6) and, also, *in vivo* (7). Although some other cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) have partial HSF activities, IL-6 is currently considered to be the major and essential HSF (3-7). Thus, in order to understand the molecular mechanism of APP induction, it is necessary to identify IL-6 responsive *cis*-acting elements (IL-6REs) and *trans*-acting factors.

Alpha-2-macroglobulin (α_2M), a plasma glycoprotein that functions as a protease inhibitor, is a major and typical APP in rats and has been studied as a good system for understanding the acute phase reaction at molecular level. Hybridization analysis using a rat α_2M cDNA probe revealed that the amount of α_2M mRNA increases remarkably during the acute phase of experimental inflammation

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(8-10). The entire structure of a chromosomal gene encoding α_2M has been elucidated (11), and several possible regulatory signals have been pointed out (12-15). In particular, the sequence (T/A)T(C/G)TGGGA(A/T) is of great interest, that was originally identified as a conserved sequence in the 5'-flanking regions of α-, β- and γ-fibrinogen genes (16). The sequence is, as we pointed out previously (12), conserved not only in fibrinogen genes but also in the upstream regions of various APP genes (17-21) including α_2M gene. Thus, it is conceivable that the sequence can act as a regulatory signal for acute phase gene induction.

In this study, we have identified an IL-6RE of rat α_2M gene by transient expression assays and showed that the conserved motif mentioned above has, in fact, a crucial role in IL-6 induced expression of α_2M gene. Futhermore, analysis of nuclear proteins binding to the IL-6RE suggested that two similar but distinct factors interact with the element in normal (non-induced) and inflamed (induced) stages.

MATERIALS AND METHODS

Transient Expression Assays

The *Eco*RV-*Bgl*II, *Rsa*I-*Bgl*II, *Eco*RI-*Bgl*II and *Pvu*II-*Bgl*II fragments from the 5'-flanking region of the α_2M gene (see Fig. 1) were fused with bacterial chloramphenicol acetyltransferase (CAT) gene derived from pSV2-CAT (22) on pUC18 (23). Site-directed mutagenesis were performed as essentially described by Inouye and Inouye (24). Each construct (13μg) was transfected together with 2μg of pRSV/L (25) into HepG2 cells ($2x10^6$) by calcium-phosphate method. Recombinant IL-6, which was obtained from Dr. Kohase (National Institute of Health, Japan) (26) or from Genzyme Corp. (Boston,MA), was added to the culture (100U/ml) at 28 hours after transfection. CAT activities were measured at 48 hours after transfection (22) and normalized by the activities of luciferase (25) derived from co-transfected pRSV/L.

Preparation of Nuclear Extracts

Male Wister rats used were obtained from the pathogen-free room of Animal Center of Fukuoka University School of Medicine. For preparation of inflamed stage extracts, rats were injected subcutaneously with turpentine oil (0.5ml/100g body weight) at 12 hours before sacrifice. Induction of α_2M gene expression was confirmed by Northern blot hybridization. Livers were homogenized in 2.2M sucrose and 3.3mM $CaCl_2$, and centrifuged at 40,000g for 60 min at 4°C (13). Pelleted nuclei were washed with buffer C (20mM Hepes-NaOH (pH 7.6), 1.5mM $MgCl_2$, 0.2mM EDTA, 1mM DTT, 25 (v/v)% glycerol and 0.1mM p-amidinophenyl methanesulfonyl fluoride (pAPMSF)) containing 0.1 (v/v)% Triton X-100, and extracted with 2.5 pellet

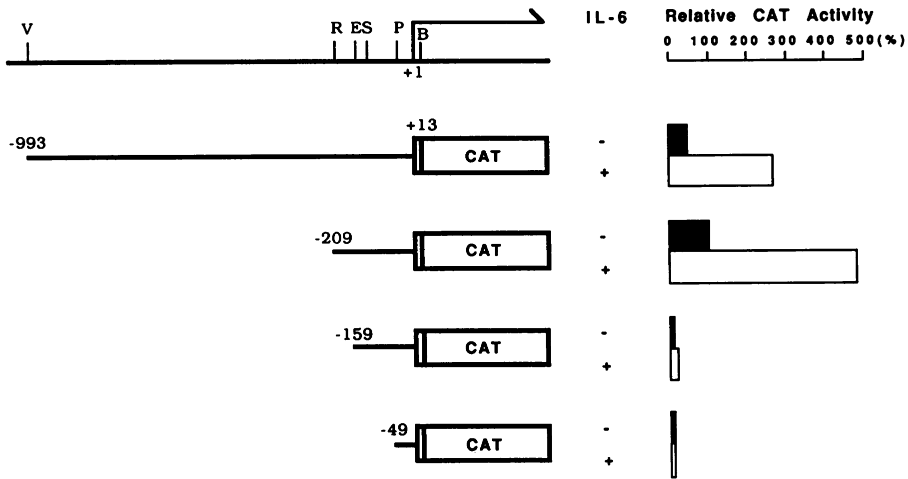

Figure 1. IL-6 responsiveness of rat α_2M-CAT fusions having progressive deletions at the 5'-flanking regions. The 5'-deletion fusions were constructed and transfected into HepG2 cells, and the CAT activities were measured. The restriction map of α_2M gene promoter region is shown in the top. Arrow indicates the major transcriptional starting site (15). V: *Eco*RV, R: *Rsa*I, E: *Eco*RI, S: *Sty*I, P: *Pvu*II, B: *Bgl*II. Filled and open blocks indicate relative CAT activities in mock and IL-6 treated cells, respectively. The CAT activity derived from the construct bearing 209 bp of upstream sequence in mock-treated cells is arbitarily taken as 100 %. Each activity represents the average of several experiments.

volume of buffer C containing 0.42M NaCl for 30 min on ice (27). After centrifugation at 100,000g for 30 min at 4°C, the supernatant was dialyzed against buffer D (20mM Hepes-NaOH (pH 7.6), 100mM KCl, 0.2mM EDTA, 1mM DTT, 20 (v/v)% glycerol and 10μM pAPMSF) for 5-6 hours at 4°C. Insoluble materials were removed by centrifugation and the supernatant was stored at −80°C until use.

Electrophoretic Mobility Shift Assays

Standard reaction mixture (20μl) for electrophoretic mobility shift assays (28, 29) contained 5-10μg of nuclear proteins, 10μg of poly (dI-dC) and a few ng of ^{32}P-labelled DNA fragments in buffer B (10mM Hepes-NaOH (pH7.6), 5mM Tris-HCl (pH7.6), 50mM KCl, 5mM $MgCl_2$, 0.1mM EDTA, 0.5mM DTT, 10 (v/v)% glycerol and 5μM pAPMSF). After standing for 20 min at room temperature, the mixture was subjected to electrophoresis on a 5% nondenaturating polyacrylamide gel containing 44.5mM Tris, 44.5mM boric acid and 1mM EDTA that had been prerun. The gel was dried after the run and exposed to an X-ray film.

DNase I Footprinting

For DNase I footprinting (30), binding reaction was processed as described above, except that 5 times or more nuclear proteins were used. Appropriate amount

of DNase I was added to the mixture, which was further kept at room temperature for 2 min. Stop solution was added to give 20mM EDTA, 0.5 % SDS and 50 µg/ml proteinase K. After incubating the mixture at 37° for 30 min, DNA was recovered by organic solvent extraction and ethanol precipitation, and applied to an 8 % sequencing gel.

RESULTS

Identification of an IL-6RE in α_2M Gene

In order to identify IL-6REs in α_2M gene, a series of transient expression assays were performed, in which a set of chimeric plasmids having progressively deleted 5'-flanking regions of α_2M gene fused with CAT gene were transfected into HepG2 cells. The cells were cultivated in the presence and absence of IL-6, and the CAT activities were measured (see MATERIALS AND METHODS). As shown in Fig. 1, the longest construct containing about 1 kb of upstream sequence of α_2M gene responded well to IL-6. Deletion to −209 did not affect the inducibility by IL-6. Inducibility of 5-10 fold is well coincided with the results of nuclear run on assays (9, 10). However, deletion of −209 to −160 region almost completely abolished the responsiveness of α_2M gene promoter to IL-6 (Fig. 1). Further deletion did not recover the inducibility. Thus, an IL-6RE was mapped to −209 to −160 region. Recently, a similar experiment was performed by Kunz *et al.* (31), who concluded that −404 to −165 region (that is −399 to −160 region in our numbering system employing the major capping site determined by Northemann *et al.* (15).) is required for the gene to be induced by IL-6. Our results showed that −209 to −160 region is sufficient for the gene to respond to IL-6. This region is also required for basal level expression of α_2M gene, because deletion of this region caused a drastic decrease in relative CAT activity in mock-treated cells (Fig. 1).

It should be noted that the IL-6RE of α_2M gene (−209 to −160 region) does contain the sequence TTCTGGGAA that is well conserved among the 5'-flanking regions of various APP genes (12). To test its functional significance, we introduced mutations into the sequence (Fig. 2). The construct bearing mutations in the sequence showed a drastically reduced responsiveness to IL-6 (Fig. 2). The conserved sequence is thus a critical and essential component of the IL-6RE. We term it IL-6RE core sequence. To our interest, the core sequence also contributed to the basal level transcription because the mutations reduced the basal level expression remarkably. A sequence completely identical to the IL-6RE core of α_2M gene is present in the IL-1/IL-6 responsive enhancer region of α_1-acid glycoprotein gene (32).

We have also noticed that the IL-6RE of α_2M gene contains an IL-6RE core-like

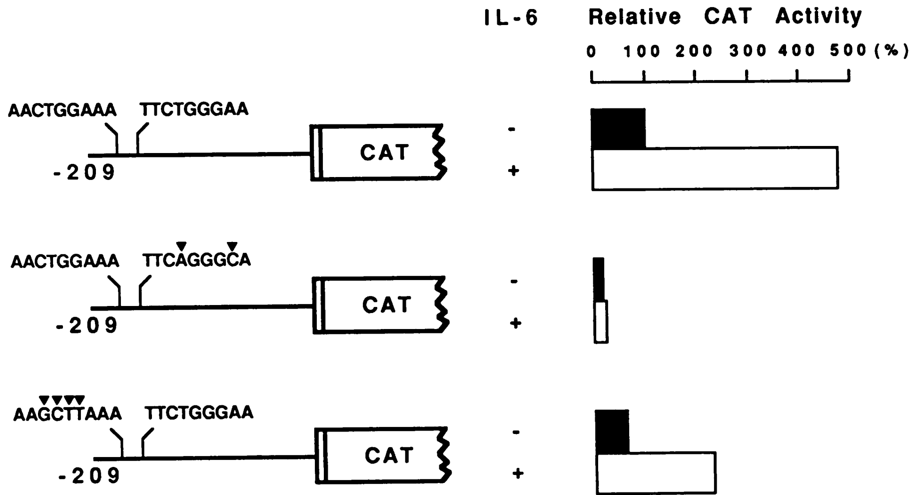

Figure 2. IL-6 responsiveness of rat α_2M-CAT fusions bearing mutations. The IL-6RE core sequence TTCTGGGAA was changed to TTCAGGGCA and the core-like sequence AACTGGAAA was changed to AAGCTTAAA by site-directed mutagenesis, and tested for IL-6 responsiveness as Fig. 1.

sequence (AACTGGAAA) at −186 to −178. The sequence shares a significant homology (eight out of nine bases) with a sequence appearing in the regulatory region of human haptoglobin gene (TACTGGAAA) (33). Recently, the sequence in the haptoglobin gene has been shown to play a crucial role for the gene to respond to IL-6 by linker scanning mutant analysis (34). It is thus conceivable that its homolog in α_2M gene also has some functional role. To test this possibility, a mutant of the core-like sequence was constructed and tested for IL-6 responsiveness. The mutation caused only a slight reduction of the inducibility by IL-6, although the net promoter activity was reduced to about 50% (Fig. 2). These results indicate that the proximal core sequence is more critical for IL-6 responsiveness than the distal core-like sequence.

Identification of Nuclear Factors Interacting with the IL-6RE

Toward the identification of *trans*-acting regulatory factors involved in IL-6 induced APP gene expression, we have searched for nuclear proteins that bind to the IL-6RE. We have examined two kinds of liver nuclear extracts: one from normal (non-inflamed) rats and the other from inflamed rats. We call them normal and inflamed stage extracts, respectively, hereafter. As shown in Fig. 3A, electrophoretic mobility shift assays revealed that both types of extracts contained proteins that formed specific nucleoprotein complexes with DNA fragment bearing

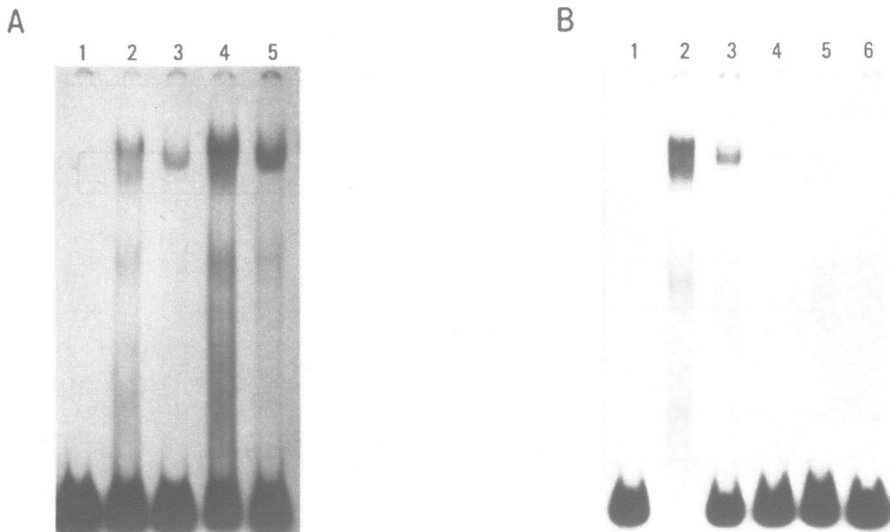

Figure 3. Electrophoretic mobility shift assays. (A) The *Rsa*I-*Sty*I fragment (see Fig. 1) was used as a probe. Lane 1, no extract. Lanes 2 and 4, 5 and 10 μg of proteins from normal stage extracts, respectively. Lanes 3 and 5, 5 and 10 μg of proteins from inflamed stage extracts, respectively. (B) The *Rsa*I-*Sty*I fragments bearing no mutation (lanes 1 to 3) and mutations in the core (lanes 4 to 6) (see Fig. 2) were used as probes. Lanes 1 and 4, no extract. Lanes 2 and 5, normal stage extracts. Lanes 3 and 6, inflamed stage extracts.

the IL-6RE. These complexes were not formed when mutations were introduced into the core sequence (Fig. 3B). Thus, these factors seemed to play some functional roles in the regulation of the expression of α_2M gene.

Binding sites of these factors were determined by DNase I footprinting. The protected regions by either extract spanned 40 bp and contained the IL-6RE core and core-like sequences (Fig. 4). The footprints seemed to be composed of tandem array of 20 bp footprints, although the upstream footprints were somewhat weaker than downstream ones, suggesting that the same or very similar factors tandemly bind to the IL-6RE core and core-like sequences.

To confirm this point, electrophoretic mobility shift assays were carried out using oligonucleotide probes. As shown in Fig. 5A, the nucleoprotein complexes were formed with the oligonucleotide containing only the core sequence (oligo-C), although the efficiency of complex formation was reduced. The complex formation was inhibited not only by the cold oligo-C but also by the cold oligonucleotide bearing only the core-like sequence (oligo-CL) (Fig. 5A). When the oligo-CL was used as a probe, similar results were obtained (data not shown). Both oligo-C and -CL

also abolished the complex formation with the *Rsa*I-*Sty*I probe that has both core and core-like sequences (Fig. 5B). Therefore we assumed that the same or quite similar factors can interact with the core and core-like sequences and that the core has higher affinities to these factors than the core-like sequence.

Since the nuclear factors interacting with the IL-6RE were identified both in the normal and inflamed stage extracts, it is interesting to ask whether or not these factors are identical to each other. It should be noted that the mobilities of complexes formed by the normal and the inflamed stage extracts were not identical (Fig. 3 & 5). The complex formed by the normal stage extract migrated more slowly than the one by the inflamed stage extract and was accompanied by a weak rapidly-migrating species. Furthermore, a close inspection of footprints revealed a small but reproducible difference in cutting pattern of lower strand around −150 region between the two extracts (see arrow in Fig. 4). These results suggested that the nuclear factors interacting with the IL-6RE at normal and inflamed stages are very similar but distinct from each other.

DISCUSSION

We have identified an IL-6RE of rat α_2M gene by transient expression assays. The identified IL-6RE contains the sequence conserved among the 5'-flanking regions of various APP genes (12). Introduction of mutations into the sequence resulted in remarkable reduction of the IL-6 responsiveness of the α_2M gene promoter, indicating the functional significance of the conserved sequence which was termed IL-6RE core. A sequence identical to the core was found in the IL-1/IL-6 responsive enhancer region of α_1-acid glycoprotein gene (32). The homolog of IL-6RE core in human haptoglobin gene was recently shown to be one of the essential components for the gene to be induced by IL-6 (34). A DNA segment responsible for acute phase induction of human C-reactive protein gene also contains a homolog of the IL-6RE core sequence (35). It is thus quite conceivable that the homologs of the IL-6RE core in other APP genes have regulatory roles in IL-6 induced gene expression.

We have also analyzed nuclear factors interacting with the IL-6RE. The factors that we have identified in livers of normal and inflamed rats bind to the proximal IL-6RE core sequence, which has been shown to be critical, with high affinity and to the distal core-like sequence, which has been shown to be less critical, with lower affinity. The distal core-like sequence might be required to stabilize the nucleoprotein complexes because the oligonucleotides bearing only the core or core-like sequences showed much reduced efficiency in the complex formation than the DNA fragment containing both of them (Fig. 5A). Mutations in

A

1 2 3 4 5 6

-191

-152

-155

-194

B

-194 -152

TGAGCAGTAACTGGAAAGTCCTTAATCCTTCTGGGAATTCTGG

ACTCGTCATTGACCTTTCAGGAATTAGGAAGACCCTTAAGACC

AACTGGAAA TTCTGGGAA

******** *********

Hpt TACTGGAAA AGP TTCTGGGAA

Figure 4. DNase I footprinting. (A) Upper and lower strands were shown in lanes 1 to 3 and 4 to 6, respectively. Lanes 1 and 4, no extract. Lanes 2 and 5, normal stage extracts. Lanes 3 and 6, inflamed stage extracts. Arrow indicates the difference in cutting pattern between normal and inflamed stage extracts. (B)

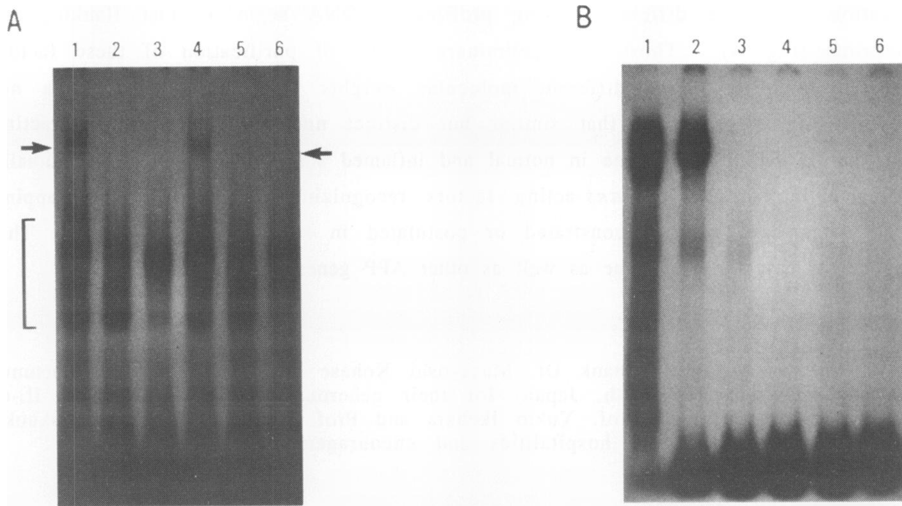

Figure 5. Electrophoretic mobility shift assays using oligonucleotides. (A) Oligo-C (TTCCTCCTTAATCCTTCTGGGAATTCTGGCTTCC) was used as a probe. Lanes 1 to 3, normal stage extracts. Lanes 4 to 6, inflamed stage extracts. Cold oligo-C and oligo-CL (GGAAGTGAGCAGTAACTGGAAAGTCCTTAAGGAA) were used as competitors (400 fold molar excess) in lanes 2 and 5, and lanes 3 and 6, respectively. Arrows indicate the positions of specific nucleoprotein complexes. Non-specific complexes were bracketed. (B) The *Rsa*I-*Sty*I fragment was used as a probe. Lanes 1, 3 and 5, normal stage extracts. Lanes 2, 4 and 6, inflamed stage extracts. Cold oligo-C and oligo-CL were used as competitors in lanes 3 and 4, and lanes 5 and 6, respectively.

the core, which caused remarkable reductions in the basal and IL-6 induced expression, abolished the binding of these factors. Thus, these factors seemed to participate in the regulation of α_2M gene expression, and to be the best candidates for *trans*-acting regulatory factors. Recently, Oliviero and Cortese identified nuclear proteins that bind to the IL-6RE core-like sequence in haptoglobin gene by electrophoretic mobility shift assays (34). Correlations between those factors and ones reported in this report remain to be elucidated.

Comparing the binding properties of these factors between normal and inflamed stages, we have noticed several differences. First, nucleoprotein complexes formed between IL-6RE (or IL-6RE core) and these factors showed different mobilities in normal and inflamed stages (Fig. 3). Second, nuclear factors in normal and inflamed stage extracts protected the same sequence from DNase I

Nucleotide sequence of the binding sites. Brackets indicate the protected regions. The IL-6RE core and core-like sequences are underlined. Homologies with sequences in other APP genes are also shown. Hpt: haptoglobin (33), AGP: α_1-acid glycoprotein (32).

digestion, but gave different cutting profiles in DNA segments just flanking the footprints (Fig. 4). Third, our preliminary results of purification of these factors revealed that they have different molecular weights from each other (data not shown). It thus seemed that similar but distinct nuclear factors are interacting with the IL-6RE of α_2M gene in normal and inflamed stages. Regulation by mutually exclusive interaction of *trans*-acting factors recognizing the same or overlapping target sites have been demonstrated or postulated in several genes (37-40). This may be the case for α_2M gene as well as other APP genes bearing IL-6REs.

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