Identification of the promoter sequences involved in the interleukin-6 dependent expression of the rat α_2 -macroglobulin gene

Dieter Kunz, René Zimmermann¹, Michael Heisig and Peter C. Heinrich^{1*}

Biochemisches Institut, Universität Freiburg, D-7800 Freiburg and ¹Institut für Biochemie, Neuklinikum Aachen, Pauwelsstrasse, D-5100 Aachen, FRG

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ABSTRACT

The 5'-region of the rat α_2 -macroglobulin gene has been characterized. A 5.6 kb Sal I - Xba I fragment containing the first 4 exons of the α_2 -macroglobulin gene and 1.3 kb of its 5'-flanking region was sequenced. The putative transcriptional start site was determined by RNase protection and primer extension analysis. TATA- and CAAT-box equivalent sequences were found. A potential glucocorticoid receptor binding site was located on the antisense strand.

DNA sequences containing the 5'-flanking region of the rat α_2 -macroglobulin gene were linked to the gene coding for the bacterial chloramphenicol acetyltransferase and introduced into Hep G2 cells. In these transfected Hep G2 cells CAT activity could be induced by recombinant human interleukin-6. Deletion analyses have shown that the sequences between -852 and -777 as well as between -404 and -165 relative to the cap site, contain regulatory elements involved in the interleukin-6 dependent induction of the α_2 -macroglobulin gene.

INTRODUCTION

Acute inflammation leads to a drastic change in the hepatic synthesis of a group of proteins, the so called acute-phase proteins [1,2]. In the rat α_2 M¹ is the major positive acute-phase protein showing increases in plasma levels of nearly hundred-fold [3-5]. This increase is preceded by a 66-fold increase in cytoplasmic mRNA [5-7]. Nuclear run-on experiments have shown that the α_2 M gene transcription is increased 4-5-fold 18 h after injection of turpentine [8] indicating that besides an increase in transcription rate post-transcriptional mechanisms may be involved in cytoplasmic α_2 M mRNA synthesis. With hypophysectomized rats we have shown that the induction of α_2 M mRNA requires the combined action of glucocorticoids and (an) inflammatory mediator (s) [9].

Mononuclear phagocytes are known to synthesize and secrete mediators such as interleukin-1 [10-13], tumor necrosis factor α [12, 14], and hepatocyte-stimulating factor [15-18] involved in the induction of acute-phase proteins. The functional and immunological identity between hepatocyte-stimulating factor from human monocytes and the B-cell-stimulatory factor 2 (also designated as interferon- β 2, interleukin-6 or 26 kDa protein) has been demonstrated [19,20]. In further studies we have been able to induce α_2 M synthesis by recombinant human interleukin-6 in rat hepatocyte primary cultures [21] as well as in vivo [22].

In order to understand the underlying mechanisms of the α_2 M gene regulation, we have isolated the α_2 M gene, and characterized its 5'-end. Chimeric plasmids containing 1.3 kb of the 5'-flanking region of the α_2 M gene linked to the gene coding for chloramphenicol acetyltransferase have been constructed and introduced into Hep G2 cells. The CAT activity of cell extracts was a measure for the transcriptional activation mediated by the whole 5'-region and a set of deletion mutants that have been generated. Using this strategy we have determined the DNA sequences that are required for the induction of the rat α_2 M gene by rhIL-6.

MATERIALS AND METHODS

Chemicals.

[α - 32 P]dATP (>15 TBq/mmol) and [γ - 32 P]ATP (185 TBq/mmol) were purchased from Amersham Buchler (Braunschweig, FRG), [14 C]chloramphenicol (>1.5 GBq/mmol) was from NEN (Dreieich, FRG). Restriction endonucleases, Klenow fragment of DNA polymerase I, SP6 RNA polymerase, T4-DNA ligase, T4 polynucleotide kinase and nuclease Bal 31 were from Boehringer (Mannheim, FRG). Reverse transcriptase from Molony murine leukemia virus and the vector pCH 110 used for cotransfections were obtained from Pharmacia (Freiburg, FRG), sequenase was from Genofit (Heidelberg, FRG). Oligo(dT)cellulose, pancreatic RNase A, RNase T₁ from aspergillus oryzae and dexamethasone were obtained from Sigma (Deisenhofen, FRG). Oligonucleotides

were synthesized by the phosphoramidite procedure [23] with a DNA synthesizer (Applied Biosystems).

Animals.

Male Wistar rats weighing about 250 g were fasted overnight prior to the RNA isolation from their livers.

Isolation of genomic clones.

A library of rat liver DNA partially digested with Hae III and cloned in the λ -phage derivative Charon 4A [24] was screened by filter hybridization [25] with an α_2 M cDNA probe [8]. Six overlapping genomic α_2 M clones were isolated by chromosome walking as described [26-28]. The isolation of one of the six clones required the screening of a genomic library constructed from DNA fragments partially digested with Eco RI (29). Since the most upstream clone contained only 165 nucleotides of the 5'- flanking region of the α_2 M gene, we have screened a genomic library constructed from DNA fragments partially digested with Sau 3A and cloned into the vector EMBL 3 [30].

DNA-sequencing.

Parts of the 5'- flanking region were sequenced by the dideoxy method of Sanger et al. (31). Defined DNA-fragments of the Sal I - Xba I 5.6 kb fragment were subcloned into the M13mp18 and M13mp19 vectors and sequenced using the sequenase system [32].

Isolation of poly(A)RNA.

Total RNA was extracted with guanidinium HCl (33) from livers of rats 18 h after intramuscular injection of 4 ml of turpentine per kg of body weight. For the isolation of poly(A)RNA oligo(dT)cellulose chromatography was used as described previously (5).

RNase protection assay.

A 32 P-labeled antisense RNA was prepared using a genomic fragment subcloned in the pSP64 vector and transcribed with SP6 polymerase. Total RNA (20 μ g) from livers of rats 18 h after turpentine injection was hybridized with the 32 P-labeled antisense RNA probe, digested with RNase A and T_1 and subjected to polyacrylamide gel electrophoresis under denaturing conditions as described by Melton et al. (34). The exact size of the

RNA fragment was determined by comparison with adjacent sequencing reactions.

Primer extension analysis.

An oligonucleotide (5'-CCGCCCTCATCCCA-3') complementary to nucleotides +15 to +29 was synthesized and used as a primer for the elongation reaction. 10-20 pmol of the synthetic oligonucleotide was labeled with [γ ³²P]ATP and polynucleotide kinase and hybridized to 10 μ g of poly(A)RNA from livers of rats 18 h after turpentine treatment. The oligonucleotide primer was elongated with cloned reverse transcriptase from Molony murine leukemia virus at 42°C for 1 h [35]. The ethanol precipitated cDNA-poly(A)RNA hybrid was redissolved in 4 μ l formamide and subjected to polyacrylamide-gel (8%)- electrophoresis and autoradiography.

Plasmid constructions.

Plasmids pUC-CAT 1 Δ -1321 and pUC-CAT 1 Δ -852 were constructed by cloning the 1.3 kb Sal I - Bgl II or the 0.85 kb Pst I - Bgl II fragments derived from the 5'- upstream sequence of the rat α_2 M gene corresponding to bases -1321 to +12 or -852 to +12, respectively, into the vector pUC-CAT 1 (Fig.4) generously supplied by Dr. G. Bornkamm (Freiburg, FRG). The other deletion mutants were obtained after cleavage of the 5.6 kb Sal I - Xba I fragment with the restriction enzymes Bcl I (-404), Eco R I (-165), Hinf I (-112) and Pvu II (-49), fill-in of the sticky ends with Klenow fragment, subsequent cleavage with Bgl II and cloning into the Sma I - Bgl II digested vector pUC-CAT 2 (Fig.4), a gift from Dr. G. Bornkamm (Freiburg, FRG). For the construction of the plasmids pUC-CAT 2 Δ -777, and pUC-CAT 2 Δ -697 the clone pUC-CAT 1 Δ -852 was linearized with Pst I, and digested with nuclease Bal 31. The reaction was stopped at different times. The purified DNA was subsequently digested with Bgl II and cloned into the Sma I/Bgl II digested vector pUC-CAT 2. Similary pUC-CAT 2 Δ -304 was constructed by linearizing pUC-CAT 2 Δ -404 with Sac I, digestion with nuclease Bal 31 followed by cleavage of the purified DNA with Bgl II and cloning into the vector pUC-CAT 2. The clones were identified by sequencing the plasmids. The plasmid DNA was prepared by alkaline lysis followed by two

centrifugation steps through CsCl to isolate supercoiled plasmid DNA [36].

Transfection of eukaryotic cells and assays for CAT and β -galactosidase activities.

Hep G2 cells used in transfection experiments were grown at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Approximately 2x10⁶ cells per dish (10 cm diameter) were transfected with 16 μ g of supercoiled CAT-plasmid to be tested and 4 μ g of plasmid pCH 110 (coding for β -galactosidase activity) by the calcium phosphate coprecipitation technique [37]. The cells were allowed to recover from the transfection for 20 h and subsequently incubated with 100 units per ml of rhIL-6 for 24 h. The adhering cells were harvested, suspended in 0.5 ml of 0,25 M Tris/HCl buffer, pH 7.5, sonicated twice for 10 seconds each and heat-treated at 60°C for 10 minutes. 20 μ l of cell extracts were used for the determination of protein, 150 μ l for the β -galactosidase assay (38) and 360 μ g for the CAT assay (39). CAT activity was expressed as the percentage of conversion of chloramphenicol into its acetylated forms by 1mg cell protein per 10 min. The measurement of the CAT activities was highly reproducible. We have normalized these numbers for equal transfection efficiencies. After cotransfection with the plasmid pCH 110 and determination of the β -galactosidase activity in the cell extracts, the CAT-activities were expressed as percent of conversion per mg of protein and OD₄₂₀ of 1.00.

RESULTS

Isolation of a genomic clone containing the 5'- region of the α_2 -macroglobulin gene

Starting with an α_2 M cDNA probe [8] a genomic clone was isolated first from a Charon 4A rat genomic library. Defined terminal fragments of this first and the successive clones were used for the isolation of six overlapping genomic clones spanning a region of about 50 kb [26-28]. An additional clone was isolated from a genomic library constructed from DNA partially digested with Sau 3A in the vector λ EMBL 3. A 1.9 kb Eco RI - Pst I fragment containing exon 1 plus 165 nucleotides

Fig. 1 Restriction map of the 5'- region of the rat α_2M gene. The isolation of the genomic clone containing the 5'- region of the rat α_2M gene is described in Materials and Methods. The 5.6 kb Sal I - Xba I fragment was subcloned for further restriction analysis and DNA sequencing. The sequencing strategy is indicated by the arrows. The solid blocks represent the coding sequences.

Abbreviations: B, BamH I; B1, Bcl I; B2, Bgl II; E, Eco R I; P, Pst I; S, Sal I; X, Xba I.

of the 5'-flanking region was used as a probe. Fig. 1 shows the restriction map and Fig. 2 the DNA sequence of the 5'- region of the rat α_2M gene. Its comparison with the published rat α_2M cDNA sequence [40] revealed that the first 4 exons of the α_2M gene are located in this area; exon 1 contains 63 base pairs of the 5'-untranslated region, 81 bp coding for the signal sequence and 17 bp coding for the aminoterminal portion of the mature α_2M . Exon 2, exon 3, and exon 4 contain 184, 157 and 53 bp of the coding region of α_2M , respectively.

Determination of the transcription initiation site by primer extension and RNase mapping

A combination of primer extension and RNase protection experiments was used to identify the probable transcriptional initiation site of the rat α_2M gene. For the RNase protection assay a complementary RNA transcript was prepared using the 1.9 kb Eco RI - Pst I genomic fragment containing exon 1 (Fig. 1),

subcloned in pSP64 and hybridized with total RNA from livers of rats isolated 18 h after turpentine injection. After digestion with ribonucleases A and T₁ the resistant RNA-RNA hybrid was analyzed on a denaturing polyacrylamide gel. As shown in Fig. 3A, two protected RNA fragments of 160 and 158 bases were found. Therefore, transcription appears to start 160 bases upstream from the exon 1/intron 1 boundary (asterisk, Fig. 2). A single-stranded 15-mer DNA corresponding to nucleotides + 15 to + 29 was used as a primer in the DNA elongation reaction with poly(A)RNA from turpentine-treated rats. The extended DNA product was 29 nucleotides in length (Fig. 3B). This indicates that the transcription initiation site of the α_2 M gene is localized at adenine + 1 (Fig. 2, asterisk).

TATA-box and CAAT-box equivalent sequences (CATAAA and TCAAAA, dashed boxes) are located between -25 and -30 or -90 and -95 bases upstream from the transcriptional start, respectively.

Furthermore, two potential transcriptional control elements are of particular interest: a glucocorticoid responsive element upstream from the transcriptional start between -32 and -37 on the antisense strand (wavy line) and the sequence 5'-CTGGGA-3' located at position -165 to -170 first described as an acute-phase protein consensus sequence by Fowlkes et. al. [41] (solid bar).

Analysis of the rat α_2 M 5'- flanking sequence for promoter function

To determine whether the upstream region of the rat α_2 M gene does contain a functional promoter, the 1.3 kb Sal I - Bgl II fragment (Fig. 1) was cloned in front of the bacterial CAT gene using the vector pUC-CAT 1 (Fig. 4). The transcriptional activity of this rat α_2 M promoter fragment in the construct pUC-CAT 1 Δ -1321 was examined by introducing this plasmid into Hep G2 cells. Hep G2 cells were chosen, because acute-phase protein synthesis can strongly be stimulated by rhIL-6 [19, Castell et al., unpublished results]. In addition, these cells can be transfected more efficiently than rat hepatoma cell lines [42].

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Fig.2 DNA sequence of the 5'-region of the rat α_2 M gene. The 4 exons of the gene are indicated by boxes. The asterisk shows the putative transcriptional start site. TATA-box and CAAT-box equivalent sequences are indicated by dashed boxes. A possible glucocorticoid binding sites (5'- TGTCT-3)', located on the antisense strand is marked by a wavy line. The solid bar under the hexanucleotide 5'- CTGGGA-3' indicates the sequence possibly involved in mediating an acute-phase response signal [41]

As shown in Fig. 5 the plasmid pUC-CAT 1 Δ -1321 introduced into Hep G2 cells is active in directing the synthesis of CAT when the cells were stimulated with 100 units per ml of rhIL-6 or rhIL-6 + 10^{-6} M dexamethasone. Dexamethasone alone led only to a slight stimulation of CAT-activity.

To find out which sequences are necessary for the transcriptional activity of the rat α_2 M promoter fragment, deletion mutants were constructed by utilizing the restriction sites Pst I (-852), Bcl I (-404), Eco RI (-165), Hinf I (-112), and Pvu II (-49). pUC-CAT 1 Δ -852 was constructed by cloning the Pst I - Bgl II 0.85 kb fragment into the vector pUC-CAT 1 (Figs. 4,6). The following constructs pUC-CAT 2 Δ -404, pUC-CAT 2 Δ -165, pUC-CAT 2 Δ -112 and pUC-CAT 2 Δ -49 were obtained after cleavage with the respective restriction enzymes, blunt end formation using Klenow fragment, Bgl II cleavage and

Fig.3 Localisation of the transcriptional start site of the α_2M gene. (A) RNase protection mapping: Complementary RNA transcripts were prepared using the genomic 1.9 kb Eco RI/Pst I fragment (Fig.1) subcloned in pSP64, hybridized to total rat liver RNA from turpentine-treated animals and digested with RNases A and T_1 . Protected fragments were analyzed on an 8% denaturing polyacrylamide gel. The fragments of a sequencing reaction served as size markers. (B) Primer extension analysis: A synthetic oligonucleotide (15-mer) complementary to nucleotides +15 to +29 was endlabeled with [γ - ^{32}P]ATP and T4 polynucleotide kinase and incubated with 10 μ g of poly(A)RNA from livers of rats 18 h after turpentine administration. After primer extension by reverse transcriptase, products were separated on an 8% denaturing polyacrylamide gel. The fragments of a sequencing reaction served as size markers.

cloning the different fragments into the vector pUC-CAT 2. Nuclease Bal 31 digestion of pUC-CAT 1 Δ -852 and pUC-CAT 2 Δ -404 led to the constructs pUC-CAT 2 Δ -777, pUC-CAT 2 Δ -697 and

Fig.4 Vectors for the construction of hybrid molecules of the rat α_2 M promoter and the bacterial CAT gene. Grey blocks indicate coding sequences of the expressed gene, open boxes indicate the SV 40 derived splice and polyadenylation sites; wavy lines designate pUC 12 sequences.

Abbreviations: B, Bam HI; B2, Bgl II; H, Hind III; E, Eco RI; P, Pst I; S, Sal I; Sc, Sac I; Sm, Sma I; X, Xba I.

pUC-CAT 2 Δ -304 (Figs. 4,6). All the constructs were transfected into Hep G2 cells and CAT activity was measured. To exclude variations in transfection efficiency and to achieve accurate comparisons of CAT activities, the plasmid pCH 110,

Fig.5 Expression of the pUC-CAT 1 Δ -1321 construct in Hep G2 cells. 20 μ g of pUC-CAT 1 Δ -1321 DNA were transfected into Hep G2 cells as described in Materials and Methods. After 20 h the cells were incubated without (control) or with 10^{-6} M dexamethasone (dex), 100 units of rhIL-6 (IL-6) per ml of medium and with 10^{-6} M dexamethasone plus 100 units of rhIL-6 per ml (dex + IL-6). 24 h after treatment the cells were extracted and analyzed for CAT activity.

Fig.6 Analysis of the regulation of rat α_2 M CAT fusions carrying progressive deletions at the 5'-flanking sequence by rhIL-6. The 5'- deletion fusions were constructed as described in Materials and Methods. The arrow ($\rightarrow$) represents the transcriptional start site, the solid block 12 nucleotides of the coding sequence of the rat α_2 M gene. The normalized relative CAT activities of the different constructs are compared with pUC-CAT 1 Δ -1321 set as 100%.

which codes for β -galactosidase activity (43), was used as an internal marker. Each cell extract was tested for both CAT and β -galactosidase activity. The ratio of CAT to β -galactosidase activity represents a normalized measure of the CAT gene expression of a given construct independent of transfection efficiency.

Fig. 6, column on the right shows the normalized values of the CAT activities by setting the expression of pUC-CAT 1 Δ -1321 as 100%. Two major changes in CAT activities were observed: one between -852 and -777 and another between -404 and -165. This observation indicates the existence of cis-acting elements necessary for the regulation of the α_2 M gene by rhIL-6.

human haptoglobin	-145	A A C A G G G G T A T T G T T	-131
			..
human α_1 -antitrypsin	-103	A A C A G G G G C T A A G T C	-117
			
rat α_2 -macroglobulin	- 35	A A C A G . C A T A A A G T C	- 22

Fig.7 Sequence comparison of the promoter regions of the human acute-phase protein genes α_1 -antitrypsin and haptoglobin with the 5'-flanking region of the rat α_2 M gene.

DISCUSSION

α_2 M is a high molecular weight protease inhibitor and besides α_1 -acid glycoprotein the major acute-phase protein in the rat. We have recently shown in rat hepatocyte primary cultures [21] as well as in vivo [22] that α_2 M synthesis is regulated by the combined action of glucocorticoids and interleukin-6. Except for the fact that hepatic α_2 M mRNA levels increase during inflammation [5-8] little is known about the mechanisms involved in α_2 M gene expression. As a first approach we have isolated and characterized the 5'-end of the α_2 M gene.

The transcriptional start site determined by an RNase protection experiment and primer extension analysis was found to be identical with the 5'-end of the rat α_2 M cDNA recently published by Gehring et al. [40]. This is in contrast to the findings of these authors, who proposed the start site 21 ± 5 nucleotides upstream from the 5'end of their α_2 M cDNA.

Usually two consensus sequences are present in the proximal 5'-flanking region of eukaryotic genes, a TATA box about 20 to 30, and CAAT box about 40 to 110 base pairs upstream from the cap site of the gene [44]. In the case of the rat α_2 M gene we did not find these canonical sequences, but TATA- and CAAT-box equivalent sequences: 5'-CATAAAG-3' and 5'-TCAAAA-3', respectively. It should be noted that Geiser et al. [45] and Burch [46] found similar sequences in the chicken vitellogenin II gene (5'-CATAAAA-3', and 5'-GCAAAA-3'). Also Fung and Schreiber detected a TATA equivalent consensus sequence in the rat major acute-phase α_1 -protein gene [47].

The synthetic glucocorticoid dexamethasone is one essential factor for the induction of α_2 M synthesis. The consensus sequence 5'-TGTCT-3' has been found in the 5'-

flanking regions of the genes for MMTV [48-50], rat tyrosine aminotransferase [51], human metallothionein [52], chicken lysozyme [53], rabbit uteroglobin [54], and chicken vitellogenin II [55,56], and demonstrated to be a binding site for the glucocorticoid receptor complex. We have found such a consensus sequence in the 5'-flanking region of the α_2 M gene on the antisense strand at position -33 to -38. Its involvement in glucocorticoid receptor binding remains to be demonstrated.

A further sequence homology is found when the DNA sequence of rat α_2 M around the TATA-box is compared with sequences of the 5'-flanking regions of the two human acute-phase proteins α_1 -antitrypsin [57] and haptoglobin [58] (Fig. 7). In the case of α_1 -antitrypsin this region is involved in the binding of a protein designated α_1 TF-A, which is essential for the efficient transcription of the α_1 -antitrypsin gene [59].

The sequence 5'-CTGGGA-3' has been found in the 5'-promoter region of several acute-phase protein genes. Fowlkes et al. [41] were the first, who described this sequence motif in the rat α -, β - and γ -fibrinogen genes. Subsequently this consensus sequence was discovered in the promoter region of human C-reactive protein [60,61], murine serum amyloid A [62], rat α_1 -acid glycoprotein [42,63], human transferrin [64], rat α_2 -macroglobulin [65] and rat prealbumin [66]. Thus far, however, a functional role of the 5'-CTGGGA-3' sequence has not yet been demonstrated. In order to localize the sequence involved in the induction of the α_2 M gene by IL-6 we constructed chimeric plasmids of the rat α_2 M - promoter and the reporter gene CAT and performed deletion analyses of this region. From the results of these deletion experiments we conclude that cis-acting elements are located between -852 and -777 as well as between -404 and -165. In the latter case the sequence 5'-CTGGGA-3', which has been found in many acute-phase protein genes [41,42,60-66] is located between -170 and -165 base pairs. When this sequence is removed a complete loss of transcriptional activity is observed in the resulting plasmid pUC-CAT 2 Δ -165. Experiments to further define and characterize the IL-6 responsive cis-acting elements in the 5'-region of the rat α_2 M gene are in progress.

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Abbreviations

α_2 M, α_2 -macroglobulin; CAT, chloramphenicol acetyltransferase; rhIL-6, recombinant human interleukin-6.

*To whom correspondence should be addressed

+EMBL accession no. X13918

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