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IDENTIFICATION OF AN INTERLEUKIN-6 RESPONSIVE ELEMENT AND CHARACTERIZATION OF THE PROXIMAL PROMOTER REGION OF THE RAT HEMOPEXIN GENE[1,2]

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The rat hemopexin (Hx) gene was isolated and studies of its transcriptional regulation initiated. For analysis by a transient expression assay, the sequence between -2400 and +21 and sequential 5' truncates were linked to the chloramphenicol acetyltransferase (CAT) gene. HepG2 cells transfected with these CAT constructs were treated with conditioned medium of lipopolysaccharide stimulated human monocytes, interleukin-1 (IL-1) or interleukin-6 (IL-6). The activities of putative regulatory regions joined to the SV40 promoter indicated that the flanking region of the rat Hx gene from -209 to -104 contains three functional regions designated proximal regulatory regions; PRR-I (-209 to -173), -II (-178 to -158) and -III (-154 to -104). We found that PRR-II contains a different class of IL-6 responsive element (RE) from that reported for the human Hx gene, and that PRR-I and PRR-III participate in the basal expression of rat Hx in HepG2 cells.

Hemopexin (Hx), an abundant plasma protein, binds an equimolar amount of heme with an exceptionally high affinity (1). The pretranslational control of rat Hx synthesis exhibits four distinct features: it is a) hepatocyte specific, b) activated at a specific developmental stage, c) responsive to hyperoxia and d) increased during the acute phase response (APR) (2).

Rat Hx transcription is induced in H35 rat hepatoma cells by cytokines IL-6 and IL-1. Unlike that for other APR proteins (3-5) this induction is dexamethasone-independent (6). The effect of IL-6 and IL-1 on transcription has been studied for many APR proteins (7-20), including human Hx (21). The control of Hx transcription during the APR seems to be species-specific, as plasma levels of Hx in the

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[2] Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. X60006.

The abbreviations used are: APR, acute phase response; CAT, chloramphenicol acetyltransferase; CM, conditioned medium of lipopolysaccharide stimulated human monocytes; Hx, hemopexin; IL-1, interleukin-1; IL-6, interleukin-6; PRR, proximal regulatory region; RE, responsive element.

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human rise only slightly (22) while those in the rat rise up to 5-fold (Metcalfe and Muller–Eberhard, unpublished results). We are reporting here on our initial characterization of the proximal promoter region of the rat Hx gene. We found a different class of IL–6 RE in the rat Hx gene than in the human, and two regions that function in the basal expression of rat Hx in HepG2 cells.

MATERIALS AND METHODS

Construction and screening of rat genomic library: Rat liver DNA partially digested with Sau3AI was cloned into bacteriophage λEMBL–3 at the BamHI site. Recombinant λ phages were screened by the plaque hybridization method using the rat Hx cDNA probe (2).

Southern blot analysis: Genomic DNA and genomic clones were analyzed according to Sambrook et al. (23).

Sequence analysis: Overlapping deletion clones were constructed by the kilosequencing method (24). Double-stranded plasmids were sequenced by the dideoxy chain termination method using Sequenase (U.S. Biochemical Corp.).

Construction of plasmids for CAT transient assays: A BamHI–NcoI fragment of the 5' flanking region (–2400 to +21) was linked in front of the bacterial chloramphenicol acetyltransferase (CAT) gene of the plasmid pSV0cat, and designated pHx Δ–2400. A series of 5' truncated clones were constructed by the kilo sequencing method (24) or by deletion from the NcoI site at –352 of pHx Δ–2400 using exonuclease Bal31. pHx(–209/–104), pHx(–209/–158), pHx(–158/–209), pHx(–209/–173), pHx(–173/–209), pHx(–154/–104) and pHx(–104/–154) were constructed by inserting the DNA fragments with linkers into the BglII site of the plasmid pA10CAT2 (see Fig. 4). Oligonucleotide was polymerized by T4 DNA ligase and purified on nondenaturing polyacrylamide gel. The synthetic oligonucleotide (25) was:

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OL-IL6:        5' GATCCTGCCGGGAAGATAGTCTGA 3'
                  3' GACGGCCCTTCTATCAGACTCTAG 5'
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pRSV–βgal was used as the internal control for all CAT assays (25).

Cell cultures and DNA transfections: HepG2 and Hep3B cells were grown in Dulbecco's modified Eagle medium containing 10% heat–inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). For DNA transfections, HepG2 or Hep3B cells (7.5×10^5 cells) were plated on 6 cm or 10 cm dishes. After 18 hours, 12 μg of the CAT constructs or 4 μg of pRSVcat were transfected together with 1 μg or 2 μg of pRSV–βgal by the calcium phosphate precipitation method (26). The cells were induced 40 hours after transfection with 15% of conditioned medium from lipopolysaccharide stimulated human monocytes (CM), human recombinant interleukin–6 (Genzyme: 1,000 U/ml, #HIL–6–D; 100 U/ml, #1542–00) or human recombinant interleukin–1β (Genzyme: 400 pg/ml, #BIL–1–C) for an additional 24 hours, and then harvested.

CAT and beta galactosidase assays: CAT expression levels were determined by the enzyme–linked immunosorbant method (5 Prime – 3 Prime Inc.), normalized by β–galactosidase activity (27).

RESULTS

Isolation of rat Hx genomic clones and Southern blot analysis: $4x10^5$ independent recombinant clones of the λEMBL–3 library were screened with the rat Hx cDNA and seven positive overlapping clones were isolated. Southern blot analysis showed that these clones spanned the entire gene and that λgRHx7 contained the 5' flanking region (data not shown). The 3.1 kb BamHI fragment of λgRHx7 (Fig. 1A) was subcloned into pUC19 and the resultant recombinant, pHxB6, was used for sequence analysis.

Southern blot analysis of genomic DNA and the genomic clone, λgRHx7, was performed using a 700 bp NcoI(+21) – BamHI genomic DNA fragment corresponding to exons I to IV of the rat Hx gene. Detection of a single band suggests that there are no rat Hx pseudogenes or closely related genes (Fig. 1B, 1C).

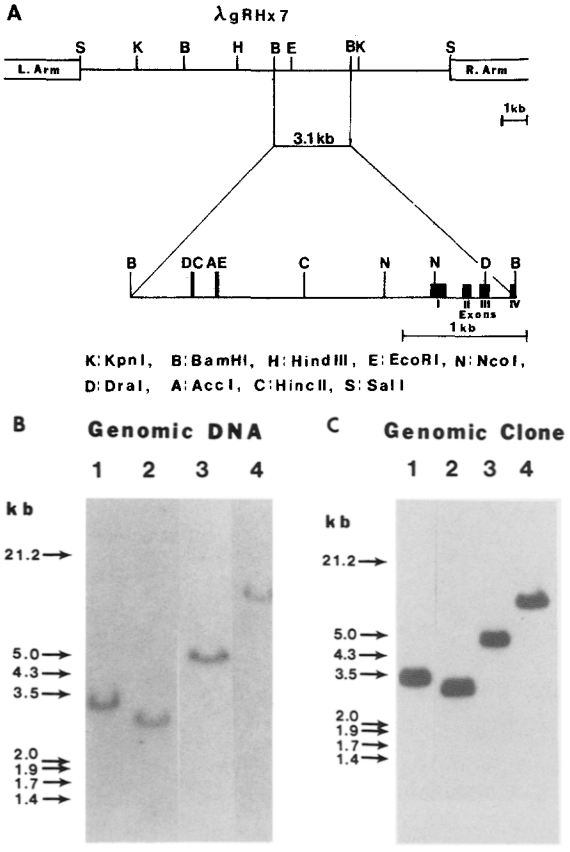

FIGURE 1. Restriction map of the rat Hx gene and Southern blot analysis. (A) The restriction map of λgRHx7 is shown as well as a detailed restriction map and the gene structure of the 3.1 kb BamHI fragment. (B,C) Twenty micrograms of rat liver DNA (B) and one microgram of the genomic clone, λgRHx7 (C), were digested with BamHI (lane 1), EcoRI + KpnI (lane 2), HindIII + KpnI (lane 3) or KpnI (lane 4) and hybridized with the labeled 700 bp NcoI(+21) – BamHI genomic fragment as a probe. The hybridizing and washing procedures were performed according to Sambrook (23). The molecular size marker is indicated at the left side of each gel.

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                                                        -828
                                                        TAAAGCATTTCTCTTCTAAGTTGGAACT

-800                                    -760
ATCAAGAGAGACTACGGGGAAGAGACACCTCCTCTTTGAGTCCTGTTACTGTCAGGCCTTCCCTCAGACACGTTTTGCAA

-720                                    -680
ATTTATTTTTCCTGGCAGGGGTTGATTTTGGGACAGGGTTTGTATTACATGACAATAGTGAGACAGCATCTCCAAACTAG

-640                                    -600
TAGTCCAGAGATAAAGCTAAAAGGTTGAGGCATATTCTCCAGAAAAGGCCTCCGTGACTGATACTTGGAAAAAGTTTTCT

-560                                    -520
TGAATGTTTCTTTTATTTATTTATTTATTTATTTATTTATTTATTCATTCATTTATTTATTGGAGCGGAGGACTGAACTT

-480                                    -440
AGGGCCTTGCATTTACTAGGCCAGTGCTCTACCACTGAGCTAAATCCCCAAACCCCTCTTGAATATTTCTGAGGAAGTTC

-400                                    -360
ATTTGTGTGTGCAGGCGTTATAGGGTGCATATAACATCTGTTTAGTTACCATGGGCTTTAGTTCAGTTGGACCTGTCCCC

-320                                    -280
TGGTACATTTGTCCCTCCTTCTGTAGAGGTGAAGGACCCCATAAGGTAACCTGAGCACCACCATTTCTTGTCAATATTCG

-240                                    -200
AGAGAGTCTCGGTGGACATGGACTGGTCACAGCTGGAAATTTGCTGAAGGGGGTAGGGATTACTGCCGGGAAGATAGTCT

-160                                    -120
GAGATTCAGGTTTCCTTTTAGACTTTGACCTTTTTTGCTGTGATGTCGTCTGCCAATATTGGCATAGTCATTGAAAGACA

-80                                     -40
AATCTTTCTCAATAGGGTGGAAAGTGGGGGTGGTGCTGTTACCAATTCTATATAAGGTCAGCCTCTTGCCCATGCTGTCC

+1
TGTGTGGTCTTTGCAGCTCGCC  ATG GCT AGG ACA GTA GTA GCA CTA AAT ATC CTG GTA TTG CTG
                         M   A   R   T   V   V   A   L   N   I   L   V   L   L
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FIGURE 2. Nucleotide sequence of the rat Hx gene 5' flanking region. The major transcription start site, numbered +1, was mapped by the primer extension method using total RNA from H35 rat hepatoma cells. The TATA and CAAT boxes are indicated by boxes. The 5' coding sequence is shown with the deduced amino acid sequence.

DNA sequence analysis: The proximal 831 nucleotides of the 5' flanking region were sequenced in both orientations. The transcription start site, determined by the primer extension method using total RNA from H35 rat hepatoma cells (data not shown), was identical to that using total liver RNA from inflammation induced or uninduced animals (2). TATA and CAAT boxes were found at –32 and –109 from the transcription start site (Fig. 2).

***Mapping of the 5' border of the cis–elements responsible for the APR*:** To identify the cis– elements required for expression during the APR, pHx Δ–2400 and successively 5' deleted CAT constructs were introduced into HepG2 cells and treated with conditioned medium (CM). The CAT level was induced 2 – 3 fold (Fig. 3) by CM in pHx Δ–2400 transfected cells, indicating that the region from –2400 to +21 contains essential information for inducibility by cytokines. The –828 deletion decreased the degree of inducibility by 40% in comparison to the -1600 deletion suggesting the presence of "a CM responsive element" between nucleotides -1600 to -828. The –252 deletion increased the CAT expression level of induced cells by 40% compared to the -519 deletion, and also increased that of uninduced cells, but to a lesser degree. This implies the existence of a negative regulatory sequence that functions in both induced and uninduced cells between nucleotides -519 and -252. The -178 deletion caused a minor decline

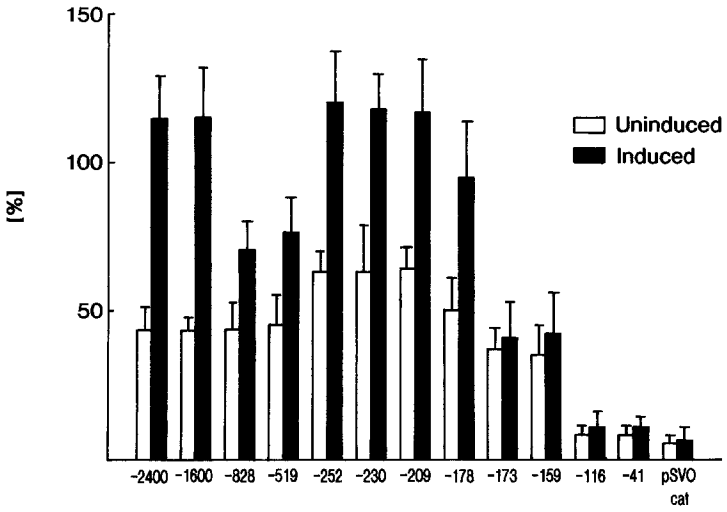


FIGURE 3. Mapping of the 5' border of the cis-regulatory element affecting an APR in the rat Hx gene. HepG2 cells were transfected with the CAT constructs containing serial 5' deletions of the rat Hx gene together with pRSV-βgal (25) by the calcium phosphate precipitation method (26). The numbers underneath the bars refer to the position of the 5' end of the Hx gene remaining in the deletion constructs. After 40 hours, transfected cells were incubated in medium with or without supplementation of the conditioned medium of lipopolysaccharide-stimulated human monocytes (CM) for an additional 24 hours. CAT expression levels were determined by the enzyme-linked immunosorbant method and normalized by the β-galactosidase activity. The expression level obtained in cells transfected with pRSVcat, a construct containing both enhancers and promoters from SV40, is referred to as 100%. Each bar represents the mean ± S.D. of at least five independent experiments.

under both conditions, and a further 5 bp deletion, to –173, abolished the inducibility. The –116 deletion drastically reduced the basal level of expression without any recovery of inducibility. Sequences from -178 to –173 were considered to contain the 5' border of the CM responsive element. The decrease in inducibility observed between –1600 and –828 suggests the presence of at least one additional cytokine responsive element.

Responsiveness of the regulatory regions involved in the APR to human recombinant IL-1 and IL-6: Seven 5' truncated CAT constructs were examined for responsiveness to recombinant cytokines (Table I). Because of the low level of induction elicited by IL-1, the presence of an IL-1 RE could not be determined. The IL-6 responsiveness, exhibited in pHx Δ–1600 to pHx Δ–178, was abolished by further deletion to –173. These results are parallel to those caused by CM, although the degree of inducibility differed (compare results of Fig. 3 and Table I), and thus establish that the 5' boundary of the IL-6 RE lies between –178 and –173.

Mapping of the 3' border of the IL-6 RE and characterization of additional elements in the proximal promoter region: To determine the 3' border of the rat Hx IL-6 RE, putative regulatory fragments linked with the SV40 promoter of the pA10CAT2 plasmid were transfected into HepG2 cells (Fig. 4). The DNA

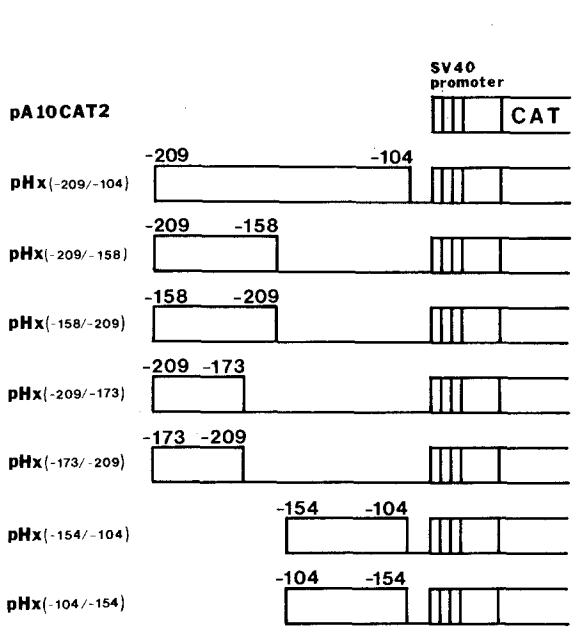
TABLE I. Responsiveness of the 5' truncated CAT constructs to IL–1 and IL–6. The DNA transfected HepG2 cells were incubated with the medium containing human recombinant IL–1 and/or IL–6. Normalized CAT levels (by β–galactosidase activity) were compared to pRSVcat as 100%. Each value represents the mean ± S.D. of at least five independent experiments. The mean of the CAT expression levels in IL–1 and/or IL–6 treated cells are compared to the uninduced cells by the Student's t test. N.D., not determined.

	CAT expression levels (%)			
Plasmids	Uninduced	IL–1	IL–6	IL–1 + IL–6
pHx Δ–1600	36.3±6.1	45.6±7.1*	54.6±4.6**	60.5±7.1**
pHx Δ–828	38.2±2.1	35.1±3.8	49.8±8.2*	46.3±2.5**
pHx Δ–209	56.5±8.0	51.5±7.8	83.4±11.8**	70.4±11.1*
pHx Δ–178	46.3±5.3	N.D.	70.0±5.6**	N.D.
pHx Δ–173	36.2±4.4	N.D.	36.5±6.8	N.D.
pHx Δ–159	38.9±8.0	33.4±5.9	41.6±6.9	40.8±5.9
pHx Δ–116	4.2±1.9	3.6±1.3	4.8±2.3	4.2±2.3

*p< 0.05, **p< 0.01.

ragment from –209 to –104 enhanced the SV40 promoter activity by eight–fold above baseline. The CAT xpression level was induced by IL–6 two–fold as compared to the uninduced level, indicating that this egion contains the IL–6 RE as well as other element(s) that affect the basal level of transcription. The ragment from –209 to –158 responded to IL–6 with a two–fold induction in both forward and inverted rientations, but the basal level of expression was raised only by 60%. 3' deletion from –158 to –173 bolished the IL–6 responsiveness yet enhanced the promoter activity in the uninduced state by two–fold bove baseline in both forward and inverted orientations. These results establish that the 3' boundary of he IL–6 RE is located between –158 and –173 and that the region from –209 to –173 contains an dditional functional element that affects basal expression. The fragment from –154 to –104 was nresponsive to IL–6 but enhanced the basal level by 3.3-fold in the forward and by 7.7–fold in the nverted direction. Thus, there is another positive transcriptional regulatory element. We designated these roximal regions: proximal regulatory regions –I (PRR–I) (–209 to –173), –II (PRR–II) (–178 to –158) nd –III (PRR–III) (–154 to –104).

RR–II contains the IL–6 RE of the rat Hx gene: To confirm that PRR–II contains the IL–6 RE, ynthetic oligonucleotides (OL–IL6 = –178 to –158) were cloned into pA10CAT2 (Fig. 5). The monomers howed little activity but the dimers induced the expression in HepG2 cells in response to IL–6 by 2– to –fold in both forwards and inverted directions. Significant amplification (16–fold induction) was btained with the hexamer (pIL6x6).



CAT expression levels in HepG2		
Uninduced	IL-6	Fold induction
1.0±0.3	1.1±0.2	1.1
8.3±1.4	16.8±2.3	2.0
1.6±0.1	3.5±0.3	2.2
1.7±0.2	3.2±0.1	1.9
2.4±0.3	2.6±0.4	1.1
2.3±0.5	2.7±0.7	1.2
3.3±0.5	3.5±0.6	1.1
7.7±0.8	8.2±0.5	1.1

FIGURE 4. Mapping of the 3' border of the IL-6 RE and characterization of the putative regulatory elements of the rat Hx promoter region. Putative regulatory regions were linked with the SV40 promoter of the plasmid pA10CAT2. The left schemes indicate the structure of each construct. Each CAT construct was transfected into HepG2 cells by the calcium phosphate precipitation method (26). For induction, HepG2 cells were treated with human recombinant IL-6. CAT expression levels were determined by the enzyme linked immunosorbant method and normalized by the β-galactosidase activity. The average of the uninduced level of the pA10CAT2 is designated as 1.0. Each value represent the mean ± S.D. of three independent experiments. N.D., not determined.

pIL6x6 was also tested in Hep3B cells, in which functional analysis of the human promoter regio had been performed (21). pIL6x6 elicited a 3.5-fold induction (data not shown) which is significan although lower in magnitude than that in HepG2 cells.

DISCUSSION

We have identified three regulatory regions in the proximal part of the rat Hx 5' flanking regio PRR-II contains an IL-6 RE, and PRR-I and PRR-III affect the basal level of transcription of the rat H gene. There are two additional regulatory regions upstream (-1600 to -828 and -519 to -252), th former region appears to be involved in APR induction and the latter to exert a negative regulatory effect.

IL-6 RE in the rat Hx gene: Since the degree of homology between the proximal promoter regions of r and human Hx is high (74%) (28), we expected that the response of rat Hx to IL-6 would be the same a that of human Hx (21). Instead, our results suggest that the transcriptional regulation of the rat gene diffe

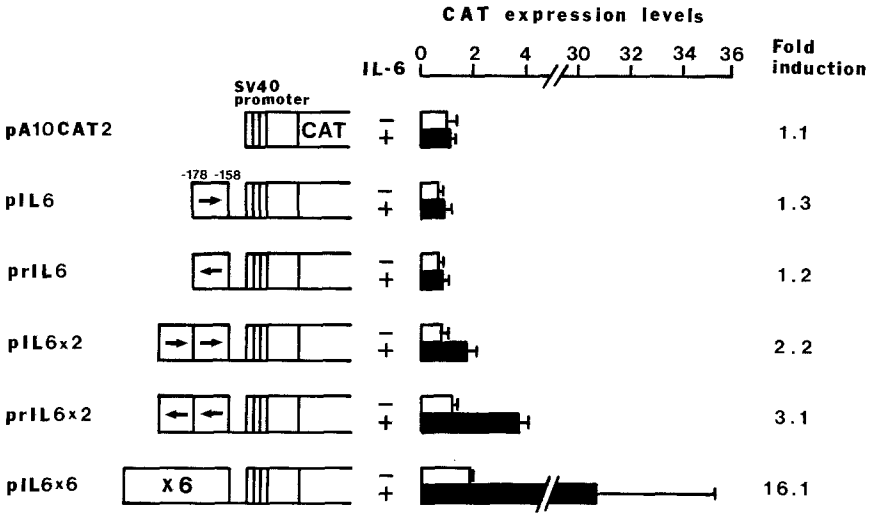


FIGURE 5. IL–6 responsiveness of synthetic oligonucleotides. Monomeric, dimeric or hexameric forms of the synthetic oligonucleotides, OL–IL6 (–178 to –158) were joined to the SV40 promoter in the plasmid pA10CAT2. The left schemes depict the structure of each construct. Each CAT construct was transfected into HepG2 cells by the calcium phosphate precipitation method (26) and treated with human recombinant IL–6. CAT expression levels were determined by the enzyme linked immunosorbant method and normalized by the β–galactosidase activity. The average of the uninduced level of the pA10CAT2 is designated as 1.0. Each bar represent the mean ± S.D. of three independent experiments. Fold induction is represented at the right side.

significantly from that of the human Hx gene during the APR. The rat IL–6 RE contained in PRR–II differs not only in location but also in sequence from the human IL–6 RE.

Two different classes of IL–6 REs have been reported. One is TNCTGGRAA, found in rat α_2–macroglobulin (13–15), rat α_1–acid glycoprotein (16,17), human (10) and rat (11,12) haptoglobin and human C reactive protein (7–9) (Table II). The rat Hx PRR-II contains "TGCCGGGAA" (–177 to

Table II. Comparison of the rat Hx IL–6 RE with that of other genes Non–conserved bases are represented in lower–case letters. Numbering is relative to the transcription start site of each gene except α1–AGP following the numbering system reported. Hx, hemopexin; MG, macroglobulin; AGP, acid glycoprotein; Hp, haptoglobin; CRP, C reactive protein.

Gene		Sequence		Ref.
rat Hx	-178	CTGCCGGGAAGATAGTCTGAG	-158	
rat α2-MG	-173	CTtCtGGGAAttctGgCTaAc	-153	13-15
rat α1-AGP	12	aTGttGG-AAttTc-------	1	16,17
	81	CTtCtGGGAAaAactggTtgt	101	
human Hp	-128	tTaCtGGaAAaAgatagTGAc	-108	10
rat Hp	-117	tTaCtGG-AAcAgtcaCTGAc	-98	11,12
human CRP	-79	aTGttGGaAAatTAtTtTaca	-69	8
Consensus		**TNCTGGRAA**		

–169), suggesting that the rat IL-6 RE belongs to this class of IL-6 RE. The other class is TTNNGNAAT, which is represented by the human Hpx-A site (21,28). The corresponding rat Hx region differs by 2 nucleotides out of the 9 invariant nucleotides from human Hpx-A (TGATGTCGT (rat IL-6 RE) and TGATGTAAT (human Hpx-A)). We are planning to perform a detailed comparison of these IL-6 RE of the rat and human Hx genes.

REs for the basal level of transcription in the rat Hx proximal promoter region: PRR–I and PRR–III function positively in the uninduced state. A particularly strong enhancing effect is exerted by PRR–III, which has a sequence element (–138 to –130) that shares homology with the promoter elements of several other genes. It is similar to e.g., two hepatoma cell specific factor binding regions on the human transferrin gene that have "TCTTTGACCT" in reverse orientations (30), and the binding site of the human α_1–antitrypsin gene for the liver specific factor, LF–A1 (32). A common 5'–TG(G/A)(A/C)CC–3' motif is found in the regulatory region of the genes for human apolipoprotein A1 and haptoglobin, and is similar to the ubiquitous COUP transcriptional factor binding site (33,34).

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