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Structure, Hormonal Regulation, and Identification of the Interleukin-6- and Dexamethasone-Responsive Element of the Rat Haptoglobin Gene

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Hepatic expression of the haptoglobin (Hp) gene in mammalian species is stimulated severalfold during an acute-phase reaction. To identify the molecular mechanism responsible for this regulation, the single-copy rat Hp gene has been isolated. The genomic sequences showed a high degree of homology with the primate Hp^1 gene. Activity of the rat Hp gene was increased in cultured liver cells by interleukin-1 (IL-1), IL-6, and glucocorticoids. The genomic Hp gene sequence spanning from −6500 to +6500, when transiently introduced into human hepatoma (HepG2) cells, directed IL-6- and dexamethasone-stimulated expression of rat Hp mRNA and protein. No response to IL-1 was detected, suggesting that the corresponding regulatory element(s) might lie outside of the tested gene sequences. An IL-6- and dexamethasone-responsive element has been localized to the promoter proximal region −146 to −55. Although the nucleotide sequences of this rat Hp gene region showed substantial divergence from that of the human gene, analysis of sequential 5′ and 3′ deletion constructs indicated an arrangement of functional IL-6 response elements in the rat Hp promoter sequence comparable to that of the human homolog. The magnitude of IL-6 regulation through the rat Hp gene promoter was severalfold lower than that of the human Hp gene. The reduced activity could be ascribed to a single-base difference in an otherwise conserved sequence corresponding to an active element in the human gene. The IL-6 response of the rat Hp element was improved severalfold by substituting that base with the human nucleotide.

Haptoglobin (Hp), a tetrameric protein of the structure $(\alpha\beta)_2$, is the major hemoglobin-binding protein in the plasma of vertebrates (11, 43). In addition to its role in clearance of free hemoglobin, the Hp-hemoglobin complex is also implicated in functioning as an inhibitor of cathepsins B and L (16). Hp is synthesized in adult but not fetal liver (10, 40). The pro-Hp form is proteolytically processed into an α and a β chain (22, 23). In humans and Old World monkeys, two closely linked Hp genes exist, of which only the upstream one is active in hepatic cells (10, 11, 40). The adjacent gene, termed haptoglobin related (Hpr), arose by gene duplication and has subsequently been modified by a 7-kilobase (kb) retroviruslike insertion into the first intron (30–32). New World primates possess only a single Hp gene (34). In humans, four structural alleles have been identified: Hp^{1S}, Hp^{1F}, Hp^2, and Hp^3 (11, 32, 39). Hp^2 and Hp^3 differ from Hp^1 by having seven rather than five exons (10, 32, 39). The difference is due to an internal duplication of exons 3 and 4, resulting in an increase of the α subunit from 83 to 142 amino acids (30, 48). Molecular and genetic information on nonprimate Hp is limited (11), although the cloning of a partial rat Hp cDNA whose sequence shows a high degree of similarity to the human Hp gene has been reported (19).

In different mammalian species, Hp belongs to the group of major acute-phase plasma proteins (28). After acute systemic tissue injury, transcription of the Hp gene, mRNA concentration, Hp protein synthesis, and plasma concentration are increased three- to sixfold (1, 33, 47). The principal acute-phase mediators for hepatic Hp regulation in human cell systems have been identified as interleukin-6 (IL-6) and glucocorticoids (7, 12, 18, 37). However, in rat cells, the combination of IL-1, IL-6, and glucocorticoids is required for maximal expression (6, 8). The hormone specificity of rat Hp gene regulation was found to be similar to that of other rat acute-phase protein genes, including those encoding α_1-acid glycoprotein (AGP), complement C3, serum amyloid A, and hemopexin, comprising the group of type I acute-phase proteins (8).

The principal *cis*-acting sequence through which IL-6 controls activity of the human Hp gene had been localized previously to the region from −180 to −50 relative to the transcription start site (40). Optimal regulation by IL-6 depends on three cooperating elements within that region (38). No information is available regarding a glucocorticoid-responsive element (GRE) in the human Hp gene.

We characterized the structural and functional properties of the rat Hp gene in order to (i) delineate the molecular basis for coordinate regulation of type I acute-phase proteins, (ii) determine the genetic base for the difference in cytokine responsiveness between human and rat genes, and (iii) assess the evolutionary conservation of regulatory elements of an acute-phase protein gene common to probably all mammals. In this paper, we report the primary structure of the cloned, single-copy rat Hp gene and demonstrate its regulation by acute-phase hormones. The lower IL-6 responsiveness of the rat Hp gene compared with that of the human gene could be ascribed to an inactive homolog of the furthest upstream human regulatory sequence.

MATERIALS AND METHODS

Cells. Reuber H-35 rat hepatoma cells (clone T-7-18) (8) were cultured in Dulbecco modified Eagle medium containing 10% heat-inactivated fetal calf serum. HepG2 cells (a gift of B. Knowles, Wistar Institute, Philadelphia, Pa.) and

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Hep3B-2 cells (a gift of G. Darlington, Texas Children's Hospital, Houston) were cultured in minimal essential medium with 10% fetal calf serum. Hormonal treatment of these cells was carried out for 18 to 24 h with serum-free medium alone or containing 1 μM dexamethasone, 100 to 250 U of human recombinant IL-6 per ml, and/or 500 U of human recombinant IL-1β per ml.

Animals. A total acute-phase reaction was induced in 3-month-old male Buffalo rats (300 g) by two subcutaneous injections of 125 μl of turpentine in the lumbar region. Control animals received 250 μl of pyrogen-free phosphate-buffered saline.

Isolation of RNA and DNA. Total liver RNA from control or acute-phase rats was extracted 24 h after treatment by the guanidine hydrochloride method as described by Labarca and Paigen (29). RNA from tissue culture cells was prepared as described by Chirgwin et al. (14). Genomic DNA from Buffalo, ACI, and Sprague-Dawley rats was isolated from nuclei essentially as described by Chapman et al. (13).

RNA analysis. For Northern (RNA) blot analysis, 15-μg samples of RNA were separated on 1.5% agarose gels containing formaldehyde, transferred to nitrocellulose, and hybridized with ^{32}P-labeled cDNA encoding rat Hp (2). After autoradiography, hybridization was quantitated by densitometric scanning and normalized to the ethidium bromide-stained rRNA bands. For primer extension, a single-stranded 20-mer (antisense sequence from bases 1757 to 1776; see Fig. 3) was synthesized at the Roswell Park Memorial Institute Biopolymer Facility by using an automated DNA synthesizer (Applied Biosystems, Inc.), purified by using oligonucleotide purification columns (Applied Biosystems, Inc.), and precipitated with ethanol. Samples of 5 μg of total liver RNA or 10 μg of tissue culture cell RNA were hybridized overnight at 45°C to 10 ng of an end-labeled primer in 0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)–0.1 M NaCl–5 mM EDTA. After ethanol precipitation, the samples were dissolved in a buffer containing 50 mM Tris, 2 mM dithiothreitol, 5 mM $MgCl_2$, 40 mM KCl, 200 μM deoxynucleoside triphosphates, 20 U of RNasin, and 25 to 30 U of avian myeloblastosis virus reverse transcriptase and incubated at 42°C for 90 min. The samples were treated with 0.2 M NaOH for 2 h at 42°C, neutralized with 0.33 M Tris, and precipitated with ethanol. After dissolving in 10 μl of 80% formamide, 5-μl samples were separated on a 20% acrylamide–8 M urea sequencing gel.

Identification and isolation of the Hp gene. The entire rat Hp gene was isolated in three overlapping lambda clones from three separate rat liver genomic DNA libraries by plaque DNA filter hybridization to ^{32}P-labeled rat Hp cDNA. λ Hp1 and λ Hp3 (see Fig. 2) were isolated from a Charon 4A library containing inserts of partial *Hae*III- and *Eco*RI-digested rat (Sprague-Dawley) genomic liver DNA, respectively (a gift of J. Bonner, Phytogen, Pasadena, Calif.). λ Hp2 was isolated from an EMBL3A library containing inserts of partial *Sau*3A-digested rat (Wistar) genomic liver DNA (generously provided by Mirjana Fogel, State University at Buffalo, Buffalo, N.Y.). The lambda inserts were subcloned into pUC13 and analyzed by restriction enzyme digestion and DNA sequencing.

Sequence analysis. Double-stranded plasmid DNA (3 to 5 μg) was denatured and sequenced by using Sequenase (U.S. Biochemical Corp.), [^{35}S]dATP (Amersham Corp.), and the dideoxy-chain termination method (45). The samples were separated on 5.5% acrylamide–8 M urea wedge gels (Bethesda Research Laboratories, Inc.). The gels were washed for 1 h in 5% methanol–10% acetic acid, dried, and exposed for 24 to 48 h.

Hp gene expression vectors. A fragment of λ Hp3, spanning from the *Eco*RI site at −6500 to the *Bgl*II site at +6500, and a fragment of λ Hp2, from the *Sal*I site at −1031 to the *Bgl*II site at +6500, were subcloned into the polylinker site of pUC13, yielding plasmids pHp(13 kb) and pHp(7 kb), respectively.

CAT plasmid constructs. Plasmids pSVOCAT and enhancerless pSV40CAT (which contains the simian virus 40 [SV40] promoter from positions 5171 to 128) (20) were used as recipients for different regions of the rat Hp gene 5′-flanking DNA (see Fig. 9). Restriction fragments comprising the region from −4100 to +26 of the Hp gene in λ Hp3 were isolated, blunt ended when necessary, and either modified by *Hin*dIII linkers and inserted into the *Hin*dIII site of pSVOCAT or modified by *Cla*I linker and inserted into the *Cla*I site of the enhancerless pSV40CAT. Promoter-proximal segments containing the IL-6-responsive element were also inserted into the polylinker site of plasmid pCT, which contains the adenovirus major late promoter attached to the chloramphenicol acetyltransferase (CAT) gene in pUC18 (a gift of D. Grayson, Georgetown Medical School, Washington, D.C.)

To create a rat Hp promoter with a human regulatory element A equivalent (38), a synthetic double-stranded oligonucleotide corresponding to the sequence −147 to −165 of the rat Hp gene (see Fig. 9), but with a guanine instead of an adenine at position −160, was added in the correct orientation 5′ to the *Sst*I site at −146 of plasmid construct 4 (see Fig. 8). The appropriate orientation, endpoints, and mutation of the HP gene inserts were verified by DNA sequencing.

Cell culture transfection and analysis. HepG2 cells were plated into collagen (Vitrogen 100; Collagen Corp.)-coated culture plates at a density of approximately 0.4×10^6 cells per 10 cm^2. After 24 h, the cells were transfected with calcium phosphate-precipitated plasmid DNA (21). For testing CAT plasmids, 3.5 μg of DNA was added to individual wells of six-well culture plates (3); for testing Hp gene expression vectors, 20 μg of DNA was added to 10-cm-diameter petri dishes. In each experiment, the DNA precipitates contained 10% (wt/wt) plasmid pIE-MUP as a marker for transfection efficiency (41) and 15% (wt/wt) pRSVGR to compensate for the glucocorticoid receptor deficiency of HepG2 cells (36). The cells were allowed to recover for 24 to 36 h before treatment with the various hormones. After 24 h, either cells were extracted and CAT activity was determined (20) or cell RNA was isolated and analyzed for rat Hp mRNA by primer extension reaction. The culture medium of the treated cells was subjected to rocket immunoelectrophoresis to quantitate the amounts of secreted human Hp (internal marker for cell responsiveness) and rat Hp and major urinary protein (MUP) (products derived from transfected genes) (3, 41). All CAT activities were normalized to the amount of MUP produced by the same cell cultures and expressed as percent conversion of substrate to product per hour per nanogram of MUP (41).

RESULTS

The rat genome has a single Hp gene. Analysis of rat liver DNA restriction enzyme digests and Southern blot hybridization, using the full-length rat Hp cDNA (2) as a probe, revealed only single hybridizing fragments (Fig. 1). The presence of additional fragments, such as in the *Bam*HI

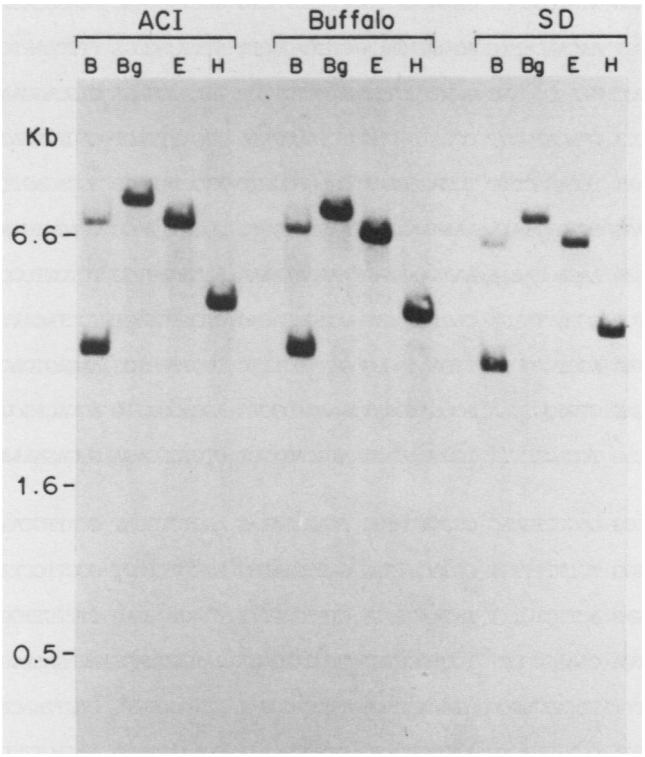

FIG. 1. Restriction digestion patterns of genomic Hp gene sequences. Liver DNA (10 μg) extracted from adult ACI, Buffalo, and Sprague-Dawley (SD) rats was digested with *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), and *Hin*dIII (H) and processed for Southern blot analysis. The filters were probed with a ^{32}P-labeled rat Hp cDNA insert. The autoradiogram was exposed for 48 h. Positions of coelectrophoresed size markers are indicated at the left.

digest, could be explained by a restriction enzyme cleavage site within the gene region. Comparison of DNA from different rat strains did not reveal any restriction fragment length polymorphisms or a change in pattern complexity. Each of the observed fragments detected in Fig. 1 could be subsequently accounted for in the cloned genomic segment (see below). Taken together, these findings suggest that only one Hp gene copy exists in the haploid rat genome. Rats, therefore, differ from most primates, which carry two to three tightly clustered Hp genes, including the most 3′-located Hpr gene, which is not expressed in liver (10, 34, 40).

Isolation and characterization of the rat Hp gene. Three overlapping clones containing Hp gene sequences were isolated from lambda libraries of rat liver genomic DNA. The total length of the cloned DNA segments spans 18.5 kb, including 6.5-kb 5′-flanking, 4.6-kb transcribed, and 7.3-kb 3′-flanking regions (Fig. 2). The locations of exon sequences were initially established by hybridization of subcloned genomic restriction fragments with segments of the Hp cDNA. The precise structure of the Wistar rat Hp gene was then determined by sequence analysis (Fig. 3). Exon-intron boundaries were derived by comparison of the genomic and cDNA sequences. The rat Hp gene consists of five exons, and its overall organization is very similar to that of the human Hp^1 alleles (Fig. 2). No Hpr gene sequences were evident in the 7-kb 3′-flanking region.

The amino acid sequence derived from the nucleotide sequence includes the 187 carboxy-terminal amino acid sequence published previously by Goldstein and Heath (19),

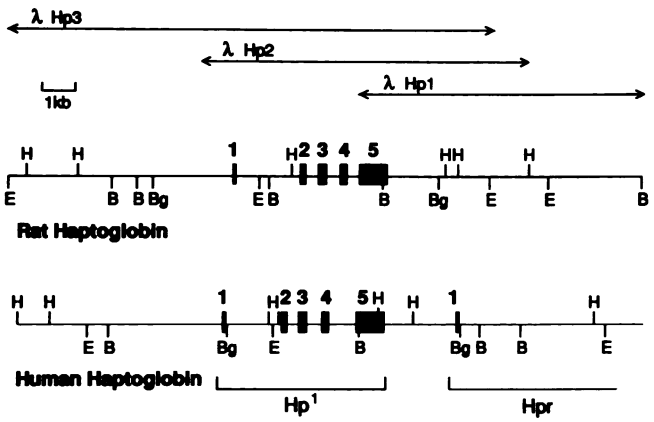

FIG. 2. Structure of the rat Hp gene. Positions of the Hp gene inserts in lambda clones (λ Hp1 to Hp3) are shown relative to the entire Hp gene segment. Restriction enzyme cleavage sites (abbreviated as for Fig. 1) and exon positions are indicated and compared with those in human Hp^1 and Hpr sequences (10, 32).

whose partial cDNA started at position 2881 (Fig. 3) and proved to be in complete agreement with ours. The predicted Hp protein is composed of an 18-residue leader peptide, 84-residue α subunit (which includes the normally removed arginine residue at the carboxy terminus [11, 27]), and 245-residue β subunit. The β subunit contains two potential N-glycosylation sites, in contrast to the human β subunit, which has four (10, 11). The rat Hp shows 75% amino acid sequence homology for the α subunit and 86% for the β subunit when compared with the human Hp^1 gene product (10).

The transcription start site was determined by primer extension analysis (Fig. 4). A single major start site 33 bases 5′ to the translation initiation codon was detected. Longer exposure of the autoradiograms revealed a minor start site 30 bases upstream of the major site. No primer extension products were detected when kidney RNA was used, demonstrating the specificity of the analysis.

Regulation of rat Hp. A 24-h acute-phase reaction resulted in a threefold increase in the concentration of Hp mRNA in rat liver (Fig. 4; 33). Moreover, the primer extension analysis indicated that no significant change in promoter utilization occurred. To assess the hormonal conditions contributing to Hp regulation, we used H-35 cells as a tissue culture test system. These cells are capable of responding to known inflammatory mediators by changing the expression of most positive rat acute-phase proteins, as is found in vivo (8). The responsiveness of the H-35 cells appeared to be more pronounced than that of hepatocytes, mainly because basal-level expression of the acute-phase proteins was extremely low. By measuring the concentration of Hp mRNA by Northern blot hybridization and the amounts of secreted Hp by immunoelectrophoresis after 24 h of treatment with optimal concentrations of hormones, we observed (Fig. 5) that in H-35 cells (i) dexamethasone had a low but detectable stimulatory effect, (ii) both IL-1 and IL-6 were strong inducers, (iii) the combination of IL-1 and IL-6 acted additively, and (iv) there was no significant synergistic effect of dexamethasone with the peptide cytokines. However, an enhanced response of H-35 cells was noted when dexamethasone was added to submaximal concentrations of IL-1, IL-6, or a combination of both (Fig. 4). In addition, primer extension analysis with H-35 cell RNA showed that the same transcription start site of the Hp gene had been used as in

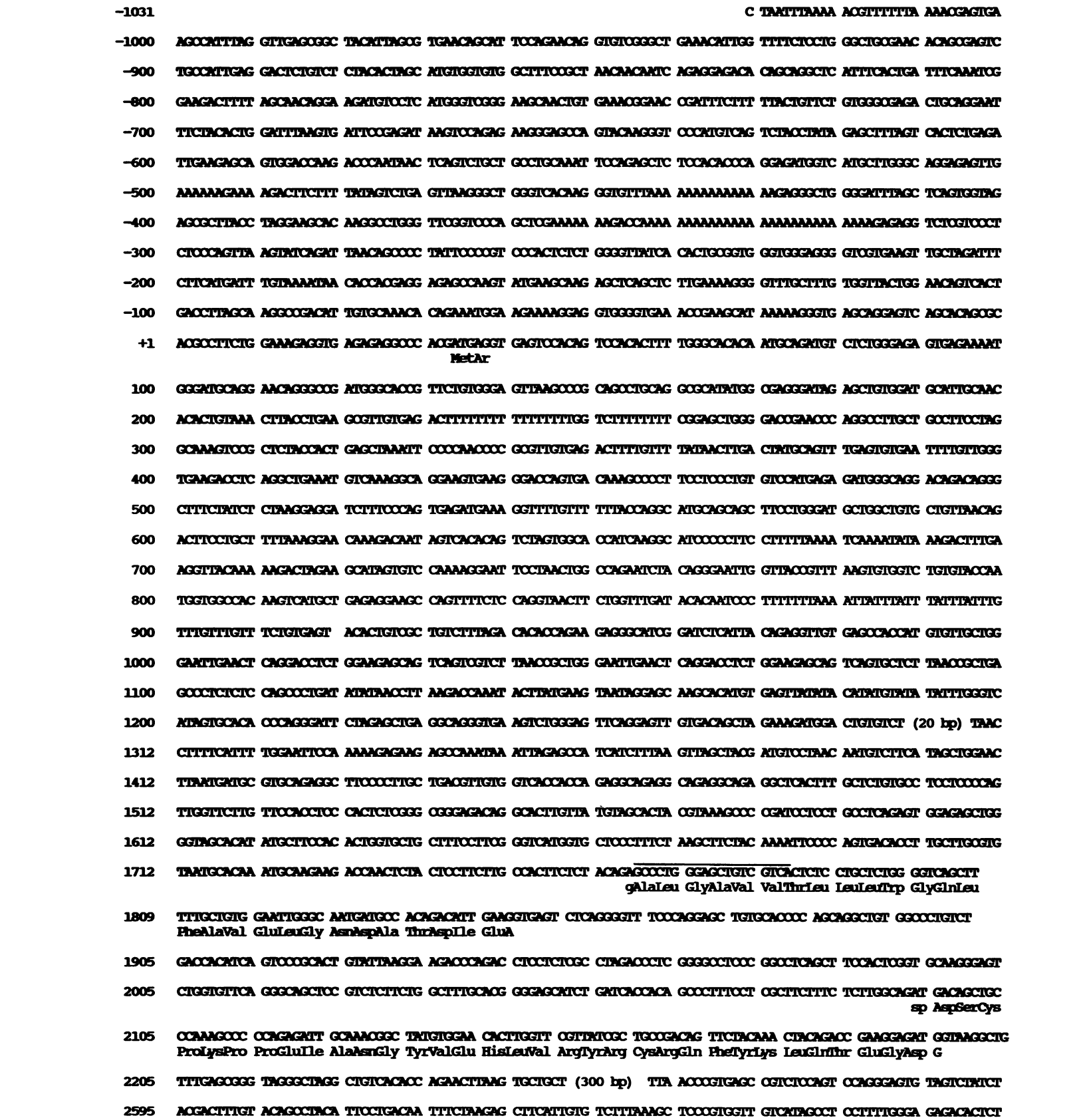

FIG. 3. Nucleotide sequence of the rat Hp gene. The primary structure of the Hp gene of Wistar rats was determined by standard techniques and included in all instances sequencing of both strands and overlapping fragments. The sequences of three small regions in introns 1, 2, and 3 could not unambiguously be determined and are therefore indicated by the estimated number of base pairs (shown in parentheses). The protein-coding regions were established by comparison with the sequence of the Hp cDNA, pIRL-9, encoding Hp from Buffalo rats (2). cDNA and genomic exon sequences are in complete agreement. Sequence of the antisense strand used for primer extension analysis (Fig. 4) is overlined; potential N-glycosylation sites are underlined.

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2695  TTAATTCCAT TTTTTCAATG AGGAAACTGA GGACGGAGAT GCCAAGGTAG CTTGTGAGGG GAAGAGTCTT GATCTGAACT CTGACCTCTT CCTGTCCAAC

2795  TCTTTCATCA GGCCACATTC ATTTTCTCTG AGCTCACCTC CTTTTGTTTC AGGAATCTAC ACCTTAAAC AGTGAGAAG CAATGGGTG AACCCAGCT
                                                                    lyIleTyr ThrLeuAsn SerGluLys GlnTrpVal AsnProAla

2891  GCTGGCGAT AAACTCCCC AAGTGTGAG GCAGGTGGGTG TTGAGGTCTT AAAGCATGGG GCTAAAATGG GGCCATGTTT CTCTTGTGTG CCTGAGTGAG
      AlaGlyAsp LysLeuPro LysCysGlu AlaV

2989  TAAGACAGGG TCAGAGAGAC ACGCTGCAAA GGAGGACAAT GACTA (290 bp) CTGCAG AGGCTCTGGA AGAATCAGCC ACCACTGCTT GCGAAACCAA

3370  CAGTACAGGA ACACTGCCCT TGCCACCTGC TCCGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG CGTGTGTATA

3470  AATATATATA TGTATATACC TACATACATA TGTAATCGTC ATCACACATA CACATTCCAC AATCCTCTTG AAAGTCAATG ACAGACCTGA AAGCTGTGTA

3570  CATTTCATTC TTAGACAAAG TTGCCCTGCA GGGGCCTGGT GTGAACTGCT GCTCACATCG GTCTCTCCTC CTCCCTCCGCA GTGTGTGGG AAGCCCAAG
                                                                                                    alCysGly LysProLys

3669  CATCCTGTG GACCAGGTA CAGCGCATC ATCGGTGGT TCCATGGAC GCCAAAGGC AGCTTTCCT TGGCAGGCC AAGATGATC TCCAGACAT GGACTCACC
      HisProVal AspGlnVal GlnArgIle IleGlyGly SerMetAsp AlaLysGly SerPhePro TrpGlnAla LysMetIle SerArgHis GlyLeuThr

3768  ACTGGGGCC ACACTGATC AGTGACCAG TGGCTGCTG ACCACTGCC CAAAACCTC TTCCTGAAT CACAGTGAG AATGCGACA GCCAAGGAC ATTGCCCCT
      ThrGlyAla ThrLeuIle SerAspGln TrpLeuLeu ThrThrAla GlnAsnLeu PheLeuAsn HisSerGlu AsnAlaThr AlaLysAsp IleAlaPro

3867  ACCTTAACA CTCTATGTG GGGAAAAAC CAGCTGGTG GAGATTGAG AAGGTAGTT CTCCACCCC GAGCGCTCT GTGGTGGAT ATCGGGCTG ATCAAGCTC
      ThrLeuThr LeuTyrVal GlyLysAsn GlnLeuVal GluIleGlu LysValVal LeuHisPro GluArgSer ValValAsp IleGlyLeu IleLysLeu

3966  AAACAGAAA GTGCTTGTC ACTGAGAAA GTCATGCCT ATCTGCCTG CCTTCCAAA GACTACGTA GCGCCAGGC CGCATGCTA TGTGTCCGG TTGGGGCGG
      LysGlnLys ValLeuVal ThrGluLys ValMetPro IleCysLeu ProSerLys AspTyrVal AlaProGly ArgMetGly TyrValSer GlyTrpGly

4065  CGGAATGTC AACTTTAGA TTTACTGAA CGTCTCAAG TATGTCATG CTGCCTGTG GCTGACCAG GAGAAGTGT GAGCTGCAC TATGAGAAA AGCACAGTG
      ArgAsnVal AsnPheArg PheThrGlu ArgLeuLys TyrValMet LeuProVal AlaAspGln GluLysCys GluLeuHis TyrGluLys SerThrVal

4164  CCTGAGAAG AAAGGCGCT GTAACTCCT GTTGGGGTA CAGCCCATC TTGAATAAG CATACCTTC TGTGCTGGC CTTACCAAG TATGAGGAA GACACTTGC
      ProGluLys LysGlyAla ValThrPro ValGlyVal GlnProIle LeuAsnLys HisThrPhe CysAlaGly LeuThrLys TyrGluGlu AspThrCys

4263  TATGGTGAC GCTGGCAGT GCCTTTGCC GTCCATGAC ACGGAGGAG GACACCTGG TATGCAGCT GGGATCCTG AGCTTTGAC AAGAGTTGT GCCGTAGCT
      TyrGlyAsp AlaGlySer AlaPheAla ValHisAsp ThrGluGlu AspThrTrp TyrAlaAla GlyIleLeu SerPheAsp LysSerCys AlaValAla

4362  GAGTATGGT GTGTATGTG AAGGCAACT GATCTGAAG GACTGGGTC CAGGAAACA ATGGCCAAG AACTAGTTC AGGGCTGACT AGAGGGCTGC ACACAGTGGG
      GluTyrGly ValTyrVal LysAlaThr AspLeuLys AspTrpVal GlnGluThr MetAlaLys Asn***

4464  GCAGGGCAAT TCACCCTGGA AGAGGAAGTA GAAGGGTTGG GGACATAATC TGAGGGCTGC TAGCCCTGCA TTGCTCAGTC AATAATAAAA AACGAGCTTT

4564  GGACC
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FIG. 3—*Continued.*

normal liver cells and that this was not influenced by the hormonal treatment. The effect of hormone treatment on Hp mRNA accumulation was detectable after 30 min and reached a maximal level after 12 h which persisted for an additional 12 h (data not shown; 8). The relative change in mRNA concentration paralleled the changes in the amounts of the secreted Hp (Fig. 5), indicating no significant influence of the hormone treatment on translational processes.

The isolated rat Hp gene is hormonally regulated. To establish whether the isolated rat Hp gene was functional and contained appropriate hormone regulatory elements, the entire gene, including 6.5-kb 5′- and 2-kb 3′-flanking regions, was subcloned into plasmid pHp(13 kb) and transiently introduced into HepG2 cells. The production and hormone sensitivity of rat Hp were assessed by immunoelectrophoretic analysis of the culture media for the transfected cells (Fig. 6A). Prominent stimulation of rat Hp production was achieved by treatment with dexamethasone and with IL-6. An additive response was observed when the two hormones were combined. IL-1, either alone or in combination with dexamethasone or IL-6, failed to exert any stimulatory effect. Regulation of the exogenous rat gene was qualitatively similar to that of the endogenous human Hp gene, as judged from the immunoelectrophoretic analysis of the culture media (Fig. 6A). As has been shown (7), production of human Hp, unlike that of rat Hp (Fig. 5), was not significantly influenced by IL-1.

Identical regulation by IL-6 and dexamethasone was observed when the 5′-flanking region of the Hp gene was reduced to 1,031 base pairs (bp), as found in plasmid pHp(7 kb) (data not shown). Primer extension analysis with RNA from cells transfected with either Hp expression vector indicated that the same major transcription start site was used as that found in rat liver [representative data for pHp(7 kb) are shown in Fig. 6B]. However, two additional transcription start sites appeared to exist at +4 and +6, which were not detected by using RNA from rat liver (Fig. 4). The transcription start sites were not influenced by the hormonal treatments. The relative change in the amounts of rat Hp mRNA, as measured by the extension analysis, was similar to that seen for Hp protein by using immunoelectrophoresis (Fig. 6).

When either pHp(13 kb) or pHp(7 kb) was transfected into mouse L cells, a basal level of expression of rat Hp was measured which was at least 10-fold higher than in HepG2 cells. Moreover, the transient production of Hp was stimulated two- to threefold by dexamethasone but was not affected by IL-1 (data not shown). From the combined data, we concluded that the cloned gene was indeed functional and that the information to respond to two of the three principal

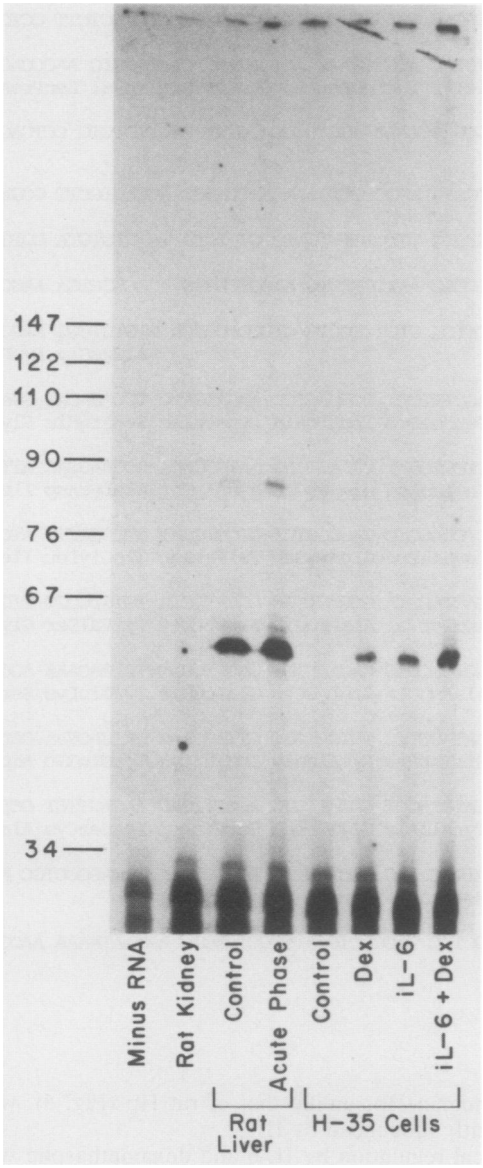

FIG. 4. Transcription start site determined by primer extension. Total cellular RNA was extracted from control rat liver and liver after 24 h of turpentine treatment and from H-35 cells treated for 18 h with medium alone, with 1 μM dexamethasone (Dex), or with 100 U of IL-6 or IL-6 plus dexamethasone. Samples of rat liver (5 μg) and H-35 cell (10 μg) RNA were used for primer extension reactions, using the primer sequence shown in Fig. 3. Autoradiograms of the electrophoretically separated products were exposed for 30 min. Positions of size markers (end-labeled, *Hpa*II-digested pBR322 fragments) are indicated in bases at the left.

rat Hp gene-regulating hormones was contained within the sequence from −1031 to +6500. The finding that the rat Hp was also active in mouse fibroblasts suggested that the functionality of the cloned gene segment in tissue culture cells was not strictly dependent on hepatocyte-specific factors.

The hormone-responsive regulatory elements are confined to a 150-bp proximal promoter region. To assess the contribution of the 5′-flanking region to the regulation of the Hp gene, the sequence from −4100 to −2 was inserted into pSVOCAT, and the resulting plasmid DNA was transfected

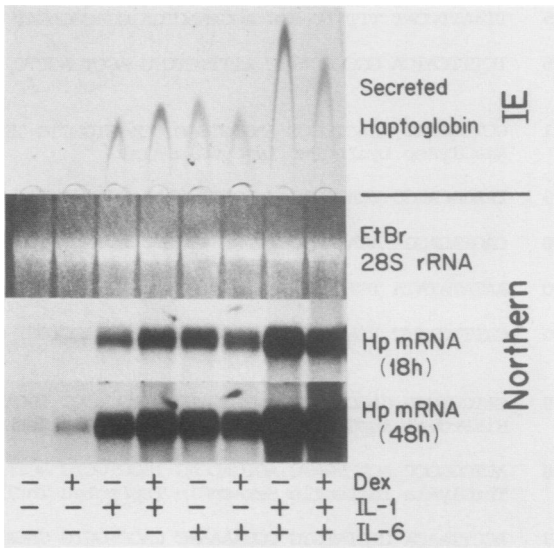

FIG. 5. Hormone-specific regulation of Hp expression in H-35 cells. Confluent monolayers of H-35 cells were treated for 24 h with serum-free minimal essential medium alone or containing 1 μM dexamethasone (Dex), 500 U of Il-1β per ml, or 250 U of IL-6 per ml. Total cellular RNA was extracted, and 15-μg samples were analyzed by Northern blotting for the amount of Hp mRNA. Ethidium bromide (EtBr) staining of the 28S rRNA and 18- and 48-h autoradiographic exposures of the Hp mRNA bands are reproduced. The amount of Hp protein secreted by each culture was determined by rocket immunoelectrophoresis (IE) of 70 μl of medium.

into HepG2 cells (Fig. 7). Dexamethasone and IL-6 alone stimulated the expression of the CAT vector, but surprisingly, the combination of the two failed to produce a significant additive action, unlike that seen for the endogenous human Hp gene or for the rat Hp expression vector (Fig. 6). As observed for the intact Hp gene (Fig. 6), IL-1, alone or in conjunction with IL-6 or dexamethasone, had no stimulatory effect. In some instances, even a slight reduction of both basal and hormone-stimulated expression was noted. The chimeric Hp-CAT construct used for Fig. 7 but without pRSVGR was transfected into H-35 and Fao cells, both of which are far less efficient in uptake and expression of exogenously supplied DNA than are HepG2 cells. However, these rat cells still yielded a qualitative regulation pattern similar to that found with human hepatoma cells, i.e., stimulation by dexamethasone and IL-6 and no stimulation by IL-1 alone or in conjunction with the other two hormones (data not shown).

Progressive 5′ deletion constructs were prepared and tested in HepG2 cells for their responses to dexamethasone and IL-6 (Fig. 8). The qualitative and quantitative regulation observed for the 4,100-bp region (construct 1) could be fully accounted for by the activity of the 145-bp proximal promoter region (construct 4). Dexamethasone stimulated the expression of the plasmids containing that sequence (constructs 1 to 4) on average two- to threefold, whereas IL-6 stimulated them four- to sevenfold. None of the constructs responded to the combination of the two hormones by a significant increase above the level for IL-6 alone.

All hormone responsiveness was lost when the 145-bp promoter fragment was reduced by an additional 59 bp (construct 6). Tests of the deleted 5′ sequences for regulatory function in combination with the heterologous, enhancerless SV40 promoter indicated that no hormone-re-

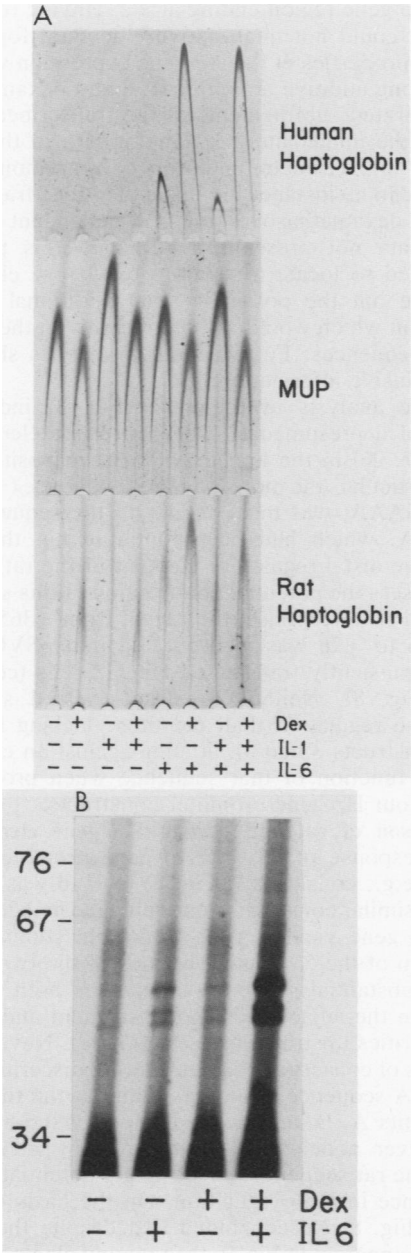

FIG. 6. Regulation of the rat Hp gene transiently transfected into HepG2 cells. (A) A 10:1:1.5 (wt/wt/wt) mixture of the plasmid DNAs pHp(13 kb), pIE-MUP, and pRSVGR was transfected into HepG2 cells in eight 10-cm-diameter dishes. After an 18-h recovery period, the cultures were treated for 24 h with 6 ml of serum-free minimal essential medium alone or with 1 μM dexamethasone (Dex), 500 U of IL-1β per ml, or 250 U of IL-6 per ml. The media were replaced, and treatments continued for 24 h. The second culture media, after removal of 50 μl for analysis of human Hp, were concentrated to 100 μl; 40 μl of each concentrate was analyzed by rocket immunoelectrophoresis for the amounts of rat Hp and MUP produced. (B) Plasmid DNA pHp(7 kb) was transfected into HepG2 cells as described above. After hormone treatment (1 μM dexamethasone [Dex]), 500 U of IL-1β per ml, or 100 U of IL-6 per ml), cell RNA was extracted and 10-μg samples were used to assess the relative amounts of rat Hp mRNA by primer extension as for Fig. 4. The autoradiograph was exposed for 18 h. Primer extension reaction with RNA from nontransfected HepG2 cells did not yield any detectable extension products. Positions of size markers (in bases) are shown at the left.

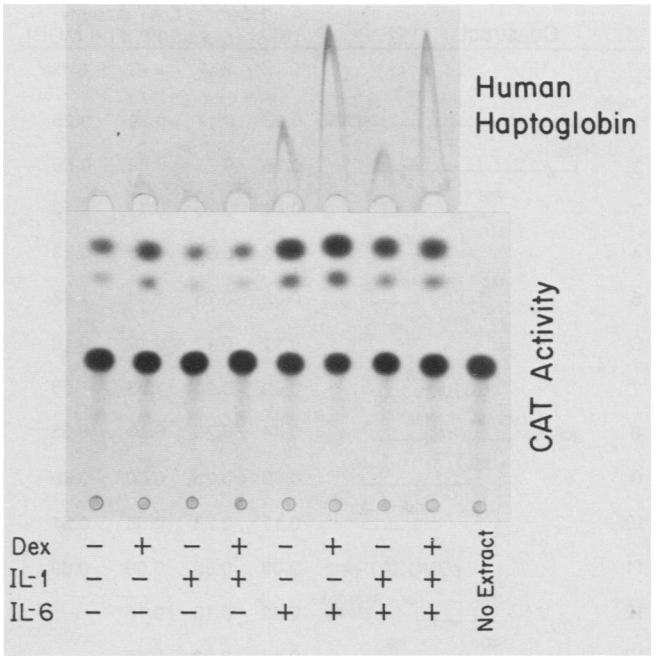

FIG. 7. Functionality of the 5′-flanking region of the HP gene in transiently transfected HepG2 cells. A mixture of the plasmid DNAs pHp(4100)-CAT, pIE-MUP, and pRSVGR was transfected into HepG2 cells in six-well plates. After a 24-h recovery period, the cells were treated for 24 h with 1 ml of serum-free minimal essential medium alone or containing 1 μM dexamethasone (Dex), 500 U of IL-1β per ml, or 100 U of IL-6 per ml. CAT activity was determined in samples of cell extracts and normalized for variations in transfection efficiency (based on analysis of MUP derived from expression of vector pIE-MUP [41]). The amount of secreted human Hp was measured by rocket immunoelectrophoresis of 50 μl of the final culture medium.

sponsive element was detectable upstream of the 145-bp active Hp promoter region (construct 12) and that the 59-bp segment, from −146 to −88, did not contain an element functional on its own (constructs 13 and 14). Even when present as a dimer, the latter 59-bp segment failed to reconstitute a hormone-responsive element, although the additional segment enhanced the overall promoter activity (construct 15). However, the 59-bp segment regained hormone-responsive function when it included an additional 32 bp of 3′-flanking sequence (construct 18). The qualitative response of that fragment was the same in the reverse orientation (construct 19), fulfilling one criterion of an enhancer. Although the region from −87 to −56 appeared to be crucial for reconstituting a function regulatory element, this sequence alone, either as a single copy or as a triplicated complex, responded only marginally to IL-6 and not at all to dexamethasone (constructs 16 and 17).

Basal-level expression of the chimeric SV40 vectors was consistently doubled when the inserted Hp gene sequences included the region from −146 to −88 (constructs 13, 14, 18, and 19). The elevated basal-level activity was also manifested when this Hp gene region was linked to a different promoter, the adenovirus major late promoter (construct 21). This enhancer activity seemed to appear when the Hp gene sequence was placed upstream of the relatively strong viral promoter but was not detectable when this sequence was present in its normal context of the weaker Hp promoter (compare constructs 4 and 6). To demonstrate that the sequence from −146 to −88 can indeed act as an enhancer

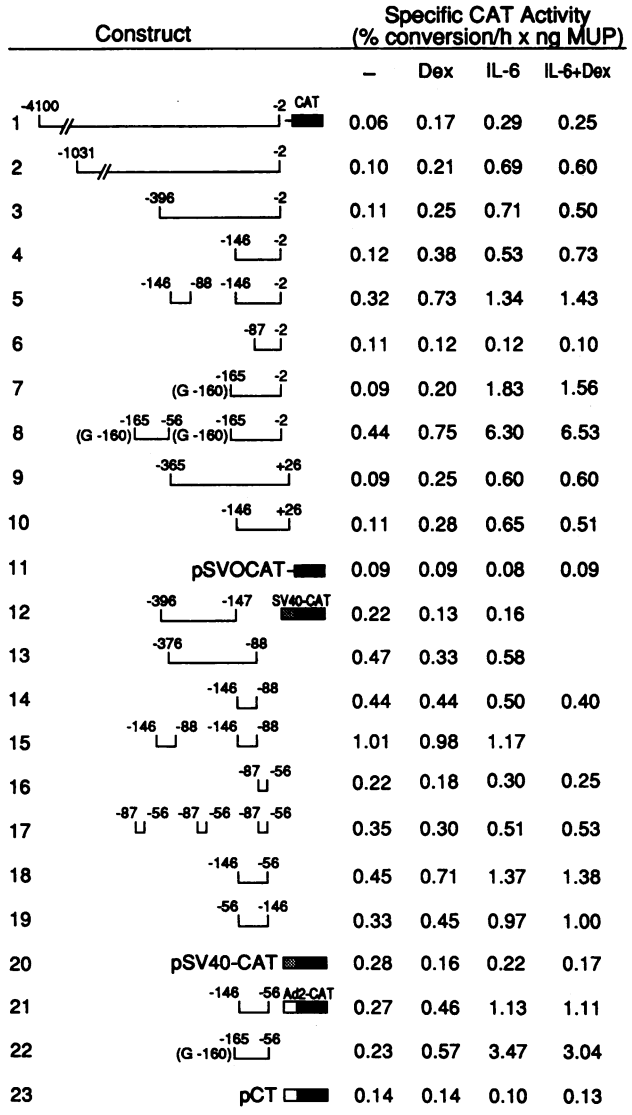

FIG. 8. Localization of the regulatory elements of the Hp gene. CAT plasmid constructs with various lengths of Hp gene 5'-flanking regions (endpoints indicated relative to the major transcriptional start sites) (constructs 1 to 10, 12 to 19, and 21 to 22) and vectors alone (constructs 11, 20, and 23) were introduced into HepG2 cells and tested for regulated expression after treatment with 1 μM dexamethasone (Dex) and 100 U of IL-6 per ml. CAT activities were normalized by relating them to the amount of MUP expressed by the same cells. Values represent means of 2 to 13 separate experiments.

on the Hp promoter, we took advantage of the observation that a dimer of it caused a further increased activity of the SV40 promoter (construct 15). Insertion of a second copy of that sequence 5' to the minimal, hormone-responsive Hp promoter region resulted in a threefold-elevated basal-level activity without altering the magnitude of the hormone response (construct 5). We concluded from these data that the sequence from −146 to −88 might be the target for a hormone-independent transcriptional factor(s) (therefore yielding improved expression of a transfected vector with a viral promoter), whereas the sequence from −87 to −56 together with the adjacent upstream sequence is required for a dexamethasone and IL-6 response.

The functional screening thus far included only the 5'-flanking Hp gene region ending at −2. Having realized that this region could not quantitatively account for all of the regulatory properties of the HP gene expression vector (i.e., lack of strong additive action of IL-6 and dexamethasone), we incorporated subfragments of the transcribed region as well as of the immediately 3'-flanking part of the Hp gene into the SV40 CAT vector and probed for hormone-sensitive expression. In no instance could we identify a fragment with significant dexamethasone- or IL-6-dependent regulatory activity (data not presented). Although this preliminary survey failed to locate a separate regulatory element, we cannot rule out the possibility that additional sequences were present which would act in concert with the promoter-proximal sequences. Future detailed analysis should yield more conclusive information.

Sequence analysis of the gene (Fig. 3) indicated the presence of a presumed IL-6-response-like element (CTG GRAA) (17, 26) in the first exon between positions +8 to +14. In particular, the more extended sequence (+5 to +14), CTTCTGGAAA, was most similar to the sequence CTTC TGGGAAA, which had been found within the IL-6/IL-1-responsive distal regulatory element of the rat AGP gene (41). To assess the potential contribution of this sequence to an elevated IL-6 response, the region from −365 to +26 or from −146 to +26 was incorporated into pSVOCAT and tested in transiently transfected HepG2 cells (constructs 9 and 10; Fig. 8). Neither construct yielded significantly greater IL-6 regulation than did those lacking the exon 1 region (constructs 3 and 4), arguing against an enhancer or regulatory function of that sequence when probed in the context of our Hp gene promoter constructs.

Comparison of rat and human Hp gene elements. The maximal response of the rat Hp gene promoter-enhancer elements (e.g., construct 3; Fig. 8) to IL-6 was sevenfold. However, similar constructs containing the first 200 bp of the human Hp gene yielded a 20- to 30-fold stimulation (40). Comparison of the 5'-flanking regions of the two Hp genes revealed substantial sequence differences both in the first exon and in the adjacent promoter segment and no significant similarities upstream of −200 (Fig. 9). Nevertheless, a few regions of conserved sequences were discernible, i.e., at the ATAAA sequence around −30 and at the three regulatory elements A, B, and C, as defined for the human gene (38). However, none of the last three regions had an identical match in the rat sequence, suggesting a potential reason for the difference in IL-6 regulation. On the basis of the data shown in Fig. 8, we concluded that despite the sequence differences apparent in Fig. 9, the rat equivalents of elements B and C seem to function as those of the human gene (see similar activities of Hp 5'Δ−153 [40] and M-157 [38]).

The rat equivalent of element A, however, was found to be inactive (compare constructs 3 and 4 in Fig. 8 and analogous human constructs Hp 5'Δ−186 and Hp 5'Δ−153 in reference 40). A single-base difference at −160 (A in rat, G in human) might cause its inactivity. To prove this point, we inserted into pSVOCAT the rat Hp sequence from −165 to −2 in which the nucleotide at −160 had been changed to a G (construct 7; Fig. 8) and tested its activity. The responsiveness of the mutant rat construct to IL-6 was greatly improved, and the magnitude of stimulation was now comparable to that reported for human Hp. An equivalently increased IL-6 stimulation was achieved when the regulatory region −165 to −56, containing the point mutation, was inserted upstream of the adenovirus promoter (construct 22). A duplication of the regulatory region, however, failed to yield an amplified hormone response (construct 8). Basal

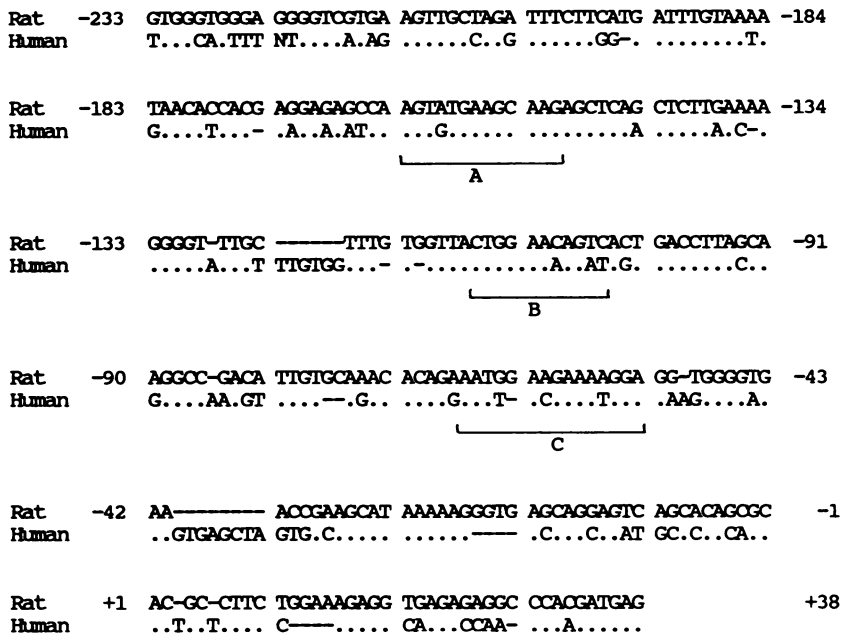

FIG. 9. Sequence comparison of the 5'-flanking and promoter regions and first exons of the rat and human Hp genes. The region from −233 to +38 of the rat Hp gene was aligned with that of the human Hp^1 gene (10, 40) according to best sequence similarity. Position of the regulatory sequences A, B, and C of the human Hp gene (38) are bracketed.

expression of the latter construct was almost fivefold elevated, probably in part because of the duplication of the potential enhancer within the region from −146 to −88.

Since all of our functional analyses of rat Hp gene elements were performed in HepG2 cells and those of the human gene were performed in Hep3B cells, we verified the compatibility of the two human cell systems by transfecting constructs 4 and 7 into Hep3B cells. CAT activities in untreated cells were 0.04 and 0.02% conversion per h per ng of MUP, whereas the activities in cells treated with 100 U IL-6 per ml for 24 h were 0.20 and 0.49% conversion per h per ng of MUP. Therefore, the magnitude of the IL-6 response of the wild-type and point-mutated Hp gene promoter in Hep3B cells was in complete agreement with the values obtained with HepG2 cells (Fig. 8). These results not only provide evidence for the crucial role of that single base for the functionality of regulatory element A (evolutionary mutant) but also suggest a likely cause for the reduced IL-6 stimulation of rat Hp gene expression. The substantially lower IL-6 response of the rat Hp gene appears to have been compensated for by responsiveness to IL-1. However, the molecular and genetic elements involved in that regulation remain to be discovered.

DISCUSSION

On the basis of exon-intron structure (Fig. 2) and encoded protein (Fig. 3), the rat Hp gene is most similar to the human Hp^1 gene (10, 32). This evolutionary evidence supports the notion that an Hp gene with Hp^1-like structure may represent the prototype for mammalian Hp genes (11, 34). The observation that rats (Fig. 1) and New World monkeys (34) have only a single-copy Hp gene, whereas Old World monkeys and humans have at least two, indicates that duplication and mutation to the Hp^2 and Hpr genes are a product of recent primate evolution. However, evolutionarily recent duplication of Hp genes is not unique to primates. A survey of several rat strains, inbred *Mus domesticus* strains, and *Mus caroli* by Southern blot analysis (as in Fig. 1) has revealed the presence of a single Hp gene in all of these rodents except for strain C57BL/6J of *M. domesticus*, which contains two genes (S. Marinković, unpublished data). Comparison of the molecular features of this murine Hp gene duplication with that of primates must await structural characterization of the mouse Hp genes.

Although in many mammalian species Hp production is stimulated after systemic injury (28), the combination of hormones that appear to be responsible for this regulation differs among species. In liver cells of rats (Fig. 5; 8) and mice (4, 42), maximal Hp expression is dependent on the combination of IL-1, IL-6, and glucocorticoid, whereas in human liver cells, the combination of IL-6 and glucocorticoids is sufficient (7, 12, 37). Because the rat and human structural genes show a high degree of sequence homology, we also expected to find a conserved arrangement and structure of the *cis*-acting regulatory elements as established for the human gene.

The cloned genomic Hp sequence, when transiently introduced into HepG2 cells, indeed responded to IL-6 and dexamethasone by increasing expression as found for the endogenous human gene (Fig. 6). However, since IL-1 did not influence the activity of the Hp expression vectors, we speculate that the IL-1 regulatory element(s) is located outside the largest sequence tested, that is, upstream of position −6500 or downstream of +6500. The fact that IL-1 and IL-6 response elements do not colocalize indicates that the Hp gene is controlled by regulatory sequences or arrangements of these sequences that are distinct from those of the AGP gene (another type 1 rat acute-phase protein gene) (8). The response of the rat AGP gene to the peptide cytokines is mediated by a single region (distal response element) located 5 kb upstream of the transcription start site (41).

The qualitative pattern of regulation by IL-6 and dexa-

methasone could be recovered in the 5'-flanking region (Fig. 8). Regulation through the 5'-flanking region differed from that of the entire Hp gene in that the two hormones did not yield a prominent additive action. A possibility is that one or more additional GREs are present in the Hp expression vector constructs and were removed during subcloning of the 5'-flanking region. However, we failed to identify a separate GRE activity by inserting subfragments of the transcribed and 3'-flanking regions in SV40 CAT vectors and testing in transiently transfected HepG2 cells. Without the support of experimental evidence, we can only speculate that such GREs, if they exist, function in the context of the promoter-proximal elements.

The overall 2- to 3-fold stimulation of all the Hp promoter-containing vectors by dexamethasone (Fig. 6 to 8) was relatively modest compared with the 15- to 50-fold stimulation achieved with the rat AGP gene element (5, 25, 44). The apparently weak activity of the Hp element might be related to the fact that the active region from −56 to −146 did not contain any sequence that was in agreement with the consensus sequence for a functional GRE (9). The precise Hp gene sequence recognized by the active glucocorticoid receptor (if there is any) has yet to be determined by appropriate protection assays.

Since the GRE function colocalized with the IL-6 response element (Fig. 8), it is possible that dexamethasone and IL-6 regulation are dependent on common transcriptional regulatory elements, such as the apparent enhancer sequence located between −146 to −88. Indeed, a strong synergistic interaction of several different transcription factors with glucocorticoid receptor function has been demonstrated (24, 35, 46). Determination of the extent to which this type of interaction applies to the glucocorticoid-dependent regulation of the Hp gene requires a detailed characterization of the IL-6 regulatory elements.

Structural and functional comparisons of the rat and human Hp gene regions involved in the IL-6 response (Fig. 8 and 9) show substantial sequence differences but nevertheless reveal an essentially identical modular arrangement of enhancer elements (enhansons [15]). The regulatory region of the human Hp gene has been found to be composed of three elements, A (−157 to −171), B (around −111), and C (−57 to −70), which, in combination with the homologous promoter, mediate an up to 30-fold-stimulated expression by IL-6 in Hep3B cells (38). By deletion and linker mutation analysis, it has been established that these elements contribute roughly equivalent activities to the overall enhancer function (38, 40). Just transferring the entire enhancer region (−186 to −38) 5' to a heterologous viral promoter caused a threefold reduction of the IL-6-specific enhancer function (ninefold stimulation by IL-6) (40). Although the individual elements (either subcloned region or synthetic oligonucleotides) were inactive when tested as single copies, a dimer of element C or a trimer of element A produced a 3.5- to 5-fold, IL-6-specific enhancement. However, a trimer of element B did not show any IL-6 response activity (38).

In contrast to the human gene, the rat Hp gene appears to contain only two functional elements equivalent to human elements B and C. As found for the human gene, 5'-flanking regions, containing the rat analog for elements A (−396 to −147), B (−146 to −88), and C (−87 to −56), failed to function as IL-6-responsive elements when tested alone (Fig. 8). Likewise, a dimer of B was inactive. Unlike the human element, an oligomerized rat element C gave only marginal IL-6 responsiveness. This discrepancy might be explained either by sequence differences within the potential protein-binding site(s) or, more trivially, by an unfavorable conformation of our oligomer construct. Regardless of this unresolved problem, the combination of the rat elements B and C reconstitutes an IL-6-responsive element that is as active as the combination of the human elements B and C. This functional similarity is surprising in view of the substantial sequence divergences between the two species (Fig. 9). However, the minimal functional sequences operative in either Hp gene must be defined before we can make conclusive statements regarding the evolutionary conservation of the functional regulatory elements B and C.

A more specific answer could be inferred from our study with respect to element A. The inactivity of the rat equivalent could be ascribed to a single-base substitution (Fig. 8). Without the synergistic action of that region, the rat haptoglobin promoter is substantially less responsive to IL-6. This might explain why the rat Hp gene is only minimally regulated by IL-6 (33). Once Hp genes of other rodents and other mammalian species are characterized, it will be possible to determine whether an active element A is a primate acquisition or whether an active element A is more general and an inactivating point mutation arose during the evolution of certain species.

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