Species-specific Changes in Regulatory Elements of Mouse Haptoglobin Genes

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Although expression of the haptoglobin (HP) as an acute phase reactant is evolutionarily conserved among mammals, there are differences among species with regard to the hormones required for stimulation. Using primary hepatocyte cultures, we show that in Mus caroli, as in rat, IL-1 and IL-6 are stimulatory, whereas in M. domesticus, as in humans, IL-1 response is diminished. In vivo, an acute inflammatory process increases hepatic HP expression in both mouse species up to 30-fold but minimally affects the low level HP expression in the lung. To define the species-specific differences in regulation, we isolated the hormone-responsive elements of the HP gene from the Mus species, M. domesticus, M. caroli, and M. saxicola. Functional studies in transfected hepatoma cells revealed an exceptionally strong dexamethasone response for all three murine HP gene elements. The IL-6 response was less prominent than in rat or human. A modest response to IL-1 was observed in M. caroli and M. saxicola. A mouse-specific insertion of a polyuridine sequence led to a binding site for the PEA3 transcription factor in the HP gene promoter of M. domesticus and M. saxicola, but not M. caroli. The specific regulatory effects of glucocorticoid receptor, C/EBPβ, and Ets proteins were documented by co-transfection.

Many characteristics of living organisms are maintained during the evolutionary process. An important question relates to the mechanisms by which such maintenance occurs. The regulation of acute phase plasma proteins in hepatic cells has emerged as an attractive experimental system for studying the molecular evolution of a gene expression phenotype (1). Genes such as for C-reactive protein (2) and serum amyloid A (3) have caught the attention of investigators because of their exceptionally high magnitude of stimulation (4, 5). In many species, the two genes are either not expressed or are unresponsive to an acute phase (5, 6). There is, however, a core set of proteins that is stimulated in most, if not all, vertebrate species. This set includes the genes encoding fibrinogen, α1- acid glycoprotein, and haptoglobin (7). During the last several years, we have focused our attention on haptoglobin (HP), since this gene appears to be co-regulated with α1-acid glycoprotein in the well-studied experimental model system of rat hepatoma cells (8) and shows an interesting species-specific pattern of regulation.

HP is synthesized in the liver as a proprotein (αβ), is processed by proteolytic cleavage into α and β subunits, and is released into the bloodstream as a tetrameric protein (α2β2) where it acts as a binding protein for free hemoglobin (9, 10). The active HP genes from human (11) and rat (12) have been cloned, and elements governing their expression and regulation have been studied in hepatoma cells. IL-6 and the functionally-related cytokines, LIF, IL-11, oncostatin M, and ciliary neurotrophic factor, stimulate HP gene transcription. This stimulation is enhanced by dexamethasone (8, 12–15). The principal regulatory elements for these effects have been localized to three regions (termed A, B, and C) within 180 bp of the transcriptional start site in the 5′-flanking region (12, 16–18). Both regions A and C serve as recognition sites for C/EBP isoforms, the binding of which mediates, in part, the activation of HP transcription (16, 18–21). Since IL-6 strongly stimulates expression of C/EBPβ in liver (22) and hepatoma cells (23), it has been postulated that haptoglobin gene regulation by IL-6 is indirect and is a consequence of an increase in C/EBP protein activity (23). Structural analysis of the rat HP gene indicated homology to the human Hp1 allele (11, 12, 24). The expression of the rat gene differs from the human gene, in that the former is stimulated not only by IL-6 and dexamethasone, but also by IL-1 or TNFα (8). The 5′-flanking region of the rat gene contains a similar arrangement of the regulatory elements A-B-C, which mediate stimulation by IL-6 and dexamethasone (12). Analysis of rat regulatory elements have thus far failed to locate the region mediating the IL-1 response of the chromosomal gene.

Although similarities in promoter architecture and functions have been demonstrated between the human and rat genes, substantial sequence alterations do exist, having the potential to contribute to the species-specific differences in regulatory pattern. Since the phylogenetic distance between human and rat is too large to make any meaningful conclusions about evolution of the regulatory elements, we have decided to compare the regulation of the HP gene among rodents, specifically the more closely related species of the genus Mus (separated from rat lineage about 15–30 million years ago; Ref. 26). We selected as representative species M. saxicola, M. caroli, and M. domesticus. The M. saxicola and M. caroli lineages diverged from the lineage leading to M. domesticus about 10 and 5 million years ago, respectively (26). In the present study, we show that substantial modifications have occurred in the promoter regions of the various mouse HP genes; some of these sequence differences may have contributed to the altered patterns of hormonal regulation observed among these species. We conclude that while induction of HP expression during the acute phase response has been maintained during mammalian evolution, the
molecular machinery mediating this induction has been altered.

MATERIALS AND METHODS

Animals—Adult 2-3-month-old males of *M. domesticus* (strain C57BL/6J) and *M. caroli* were obtained from the mouse colony at Roswell Park Cancer Institute (RPCI) (maintained by Dr. V. Chapman); *M. saxicola* were generously provided by Dr. G. Watson (The Jackson Laboratory). Stimulation of acute phase plasma protein genes was induced either by two subcutaneous injections of 25 μl of turpentine or a single intraperitoneal injection of 25 μl of saline solution containing 25 μg of lipopolysaccharides or 5 μg of dexamethasone. The animals were killed 24 h later. Serum was prepared for immunoelectrophoretic analysis of the plasma proteins, and liver, lung, and other organs were collected for isolation of RNA (27). Parts of the livers were also used to prepare DNA (28).

Tissue Culture Systems—Primary cultures of hepatocytes from *M. domesticus* and *M. caroli* were prepared and maintained as outlined previously (29). Reuber H-35 cells (clone T7-18; 8, 21, 23), HepG2 cells (30), and Hepa-1 cells (31) were cultured as described. All treatments were carried out for 24 h in serum-free minimal essential medium using the following factors at the indicated concentrations: 1 μg dexamethasone, 100 ng/ml COS cell-derived human recombinant IL-6 and LIF (Genetics Institute), 0.5 ng/ml human recombinant IL-1β (Immunex Corp.). The change in synthesis and secretion of specific plasma proteins were quantitated by rocket immunoelectrophoresis (32). The areas under the precipitation peaks were integrated and used to calculate the amounts of antigen.

RNA Analysis—Total RNA (10 μg) or polyadenylated RNA (5 μg) was separated on 1.5% agarose gels containing formaldehyde and transferred to nitrocellulose. The membranes were hybridized with 32P-labeled cDNA probes encoding mouse HP genes (positions -237 to +20) as described. The bound radioactivities were quantitated by a PhosphorImager (Molecular Dynamics) using the volume integration mode. The cap site of HP mRNAs from liver and lung were determined by primer extension analysis (37) using the antisense sequence representing a part of the second exon (GC- CCGAGCTGCTGATCA) as primer. The change in synthesis and secretion of specific plasma proteins were quantitated by rocket immunoelectrophoresis (32). The areas under the precipitation peaks were integrated and used to calculate the amounts of antigen.

CAT Reporter Gene Constructs—The promoter and 5′-flanking region of all mouse HP genes (positions -237 to +20) were inserted into the HindIII site of pOCT, which contains the enhancer- and promoterless CAT gene inserted into the HindIII-BamHI site of pUC18 and was provided by Dr. D. R. Grayson, Dept. of Anatomy and Cell Biology, Georgetown Medical School; the resulting plasmid was denoted pHP(237)OCT. The genomic SstI fragment (1300 to -196) of *M. domesticus* HP gene was inserted into the SstI site at nucleotide -196 of pHP(237)OCT. The genomic fragment containing nucleotides -237 to -105, -237 to -185, -184 to +20, and -112 to +20 of the *M. domesticus* HP gene were generated by polymerase chain reaction. The former two were inserted into pCT 5′ to the major late adenovirus promoter and the latter two into pOCT.

Cell Transfection—H-35 cells were transfected by the DEAE-dextran method (38) and HepG2 cells by the calcium phosphate method (39). Plasmid DNA mixtures were composed of the CAT reporter gene construct (5-17 μg/ml) and pIE-MUP (2 μg/ml); the latter served as a marker for transfection efficiency (40). In some experiments, expression plasmids for transcription factors (5 μg/ml) were added; these included pCD-C/EBPβ (23), pRSVGR (41), pMLP PEAK (33), pCD-C/EBPβ (42), pCD-PEAK (43), and pCD-C/EBPβ (provided by Dr. R. A. Maki, La Jolla Cancer Research Foundation) (35, 43). After overnight recovery, the transfected cell cultures were subdivided and treated for 24 h with serum-free medium containing dexamethasone and IL-6. End-labeled DNA fragments containing the HP promoter from position -239 to +20 and LIF inserted into the major late adenovirus promoter. The CAT activity was determined in cell extracts (38) and normalized to the level of major urinary protein (MUP) expressed and quantitated by immunoelectrophoresis of the culture medium (40). The values were expressed as percent conversion of chloramphenicol to acetylated products per h per ng of MUP (12, 21, 23, 25). Changes in synthesis of endogenous plasma proteins were quantitated by immunoelectrophoresis and used as a measure of the magnitude of stimulation ranging between 5- and 30-fold (Table I). Acute phase induction of HP expression in *M. caroli* and *M. saxicola* was of a similar magnitude, although basal mRNA expression appeared to be severalfold higher in these two species.

An organ survey revealed that the liver is the major site of HP expression in all species. Lung exhibited 20% of the basal liver value for *M. domesticus* and 1% for *M. caroli* (data not shown) and *M. saxicola* (Table I). Expression in lung was minimally affected by an acute phase, regardless of whether it had been elicited by tissue damage or endotoxin reaction (Table I). Only trace amounts of HP mRNA were noted in the submaxillary gland, and none was detected in kidney, spleen, heart, pancreas, small intestine, and brain (data not shown).

HP expression in the liver was stimulated roughly 2-fold by

![FIG. 1. Acute phase regulation of HP expression in *M. domesticus*. One group of three animals was left untreated (Control), and a second group was treated with turpentine (Acute Phase). After 24 h, RNA were prepared from the lung and liver of each animal and analyzed by Northern blot hybridization. The autoradiograms were exposed for 24 h. The band representing the cytoplasmic HP mRNA form is shown. The ethidium bromide staining pattern of the liver RNA is reproduced to illustrate equal RNA loading. Equal amounts of serum (0.4 μl) were analyzed for HP concentrations by rocket immunoelectrophoresis.](image)
Liver and lung RNA were analyzed by Northern blot hybridization for HP mRNA using 32P-labeled cDNA to rat HP as probe. A pool of 13 preparations of liver RNA from untreated M. domesticus served as internal standard for all analyses and has been defined as 100. The radioactivity in all analyses was quantitated by PhosphorImager scanning. Mean values ± S.D. (or range in case of duplicates) are presented.

| Treatment          | M. domesticus | M. caroli | M. saxicola |
|--------------------|---------------|-----------|-------------|
|                    | Liver    | Lung     | Liver     | Liver     | Lung     |
| None               | 100 ± 20 (13)| 20 ± 1 (3)| 510 ± 150 (6)| 620 ± 120 (6)| 6 (1) |
| Lipopolysaccharide | 920 ± 100 (4)| 30 ± 4 (2)| 3200 (1)  | 10400 (1) | 11 ± 6 (3) |
| Turpentine         | 2900 ± 300 (9)| 30 ± 3 (3)| 8200 ± 2300 (6)| ND* | ND |
| Dexamethasone      | 220 ± 20 (4)| ND       | 870 ± 390 (6)| ND | ND |

* ND, not determined.

dexamethasone (Table I), suggesting that corticosteroid action could account for only a fraction of the acute phase-mediated change in HP mRNA.

**Cytokine Stimulation of HP Expression Differences between M. domesticus and M. caroli**—To identify which of the known acute phase-associated cytokine combinations was most effective in stimulating HP in the mouse species, we measured the effects of IL-1, IL-6, and glucocorticoids in primary hepatocyte cultures. Treatment with IL-6 and dexamethasone, either alone or in combination (Fig. 2), produced similar increases in HP in both M. domesticus and M. caroli. In contrast, IL-1 stimulation was more prominent in M. caroli than in M. domesticus. In both species, maximal HP expression was observed with the combination of IL-1, IL-6, and dexamethasone. Thus, there is a distinct species difference in IL-1 regulation of HP. The overall cytokine response pattern for the M. domesticus HP gene resembled that in HepG2 cells (14) and human hepatocytes (13), whereas the pattern for M. caroli was more like rat (see upper panels in Fig. 4, below). Both mouse species showed a stronger response to dexamethasone alone than seen in rat or human.

**HP Gene Promoter**—Southern blot analysis of liver DNA from M. domesticus, M. caroli, and M. saxicola revealed a simple pattern of restriction enzyme fragments that hybridized to HP cDNA, suggesting the presence of a single copy gene per haploid genome (data not shown).2 The existence of a single HP gene was confirmed for M. domesticus by isolation of the entire HP structural gene including flanking regions. Since the available libraries for genomic DNA for M. caroli and M. saxicola did not contain HP genes, we isolated the promoter regions from these species by polymerase chain reaction. We assumed that the region between positions -260 and -240, that was found to be identical between M. domesticus and rat, was also identical in M. caroli and M. saxicola; therefore, we used an oligonucleotide corresponding to this region as the upstream primer. The first exon sequence, the downstream primer, was already known from cDNA analyses.2 The transcription start sites (+1) were determined by primer extension. An identical start site was observed for the M. caroli and M. saxicola HP mRNAs.

The comparison of promoter sequences (Fig. 3) (note that all sequence information is given relative to the M. domesticus sequence) revealed several interesting features. All HP genes contain segments of homology that coincide with the regulatory elements A and B (−210 to −140). The relative location of element C (−120 to −140) appeared to be much less conserved. Element C of M. saxicola differs from the other rodents in that a thymidine is inserted into the C/EBP binding site between −127 and −128. However, a C/EBP consensus sequence unique to the M. saxicola gene and overlapping with a PEA3 recognition sequence appeared at position −110.

The murine HP promoters were characterized by the presence of a purine-rich sequence located between −120 and −70, between the A-B-C elements and the transcriptional start site. This sequence is extensively deleted in the M. caroli, rat, and human genes. Minor modification of this polypurine stretch led to the creation of a consensus PEA3 binding site at position −90 in M. domesticus and at −110 in M. saxicola.

**Regulatory Elements in the 5′-Flanking Region of the Murine HP Genes**—To examine the function of the murine HP promoters, the 5′-flanking regions of the three mouse HP genes homologous to the M. domesticus region from −237 to +20 were inserted into pOCT, and the resulting plasmids were introduced into the cytokine-responsive human (HepG2) and rat (H-35) hepatoma cell lines. Fig. 4 shows results example for M. domesticus HP-CAT constructs. Treatment of the transfected cells with various combinations of effectors revealed that the mouse gene elements mediated an exceptionally strong response to dexamethasone in both HepG2 (Fig. 4A) and H-35 (Fig. 4B and Table II) cells. The magnitude of stimulation in HepG2 cells was surprisingly high (Table IV) considering that these cells have low glucocorticoid receptor levels (45).

The gene constructs showed a minor response to IL-6 that was somewhat higher in HepG2 cells than H-35 cells. There was a cooperative effect of IL-6 and dexamethasone primarily in HepG2 cells (Fig. 4, Table II). IL-1 or TNFa, alone or in combination with dexamethasone, proved to be ineffective on the transfected M. domesticus constructs, but was capable of activating about 2-fold the M. caroli and M. saxicola constructs (Table II). The minor IL-1 response of the latter two HP constructs probably contributed to the substantially increased CAT expression in cells treated with the combination of IL-1, IL-6, and dexamethasone.
To assess the significance of the A-B-C element-containing region in the regulation by dexamethasone and IL-6, various lengths of 5'-flanking regions and selected subfragments were tested in transfected H-35 cells (Table III). Deletion from -1300 to -237 did not alter the regulatory pattern significantly, although the dexamethasone response was consistently higher. Removal of the A element (by deletion to -184) and the B-C elements (by deletion to -112) led to a stepwise reduction in dexamethasone stimulation. The A element (-237 to -185), either in single copy or in triplicate, was ineffective as a hormone-responsive element, but in the context of the A-B-C segment (-237 to -106), a significant response to both dexamethasone and IL-6 effect was observed. This finding differs from the one reported for human analog gene constructs, in that the latter produced a severalfold higher IL-6 response.

Role of the Glucocorticoid Receptor in HP Regulation—
Dexamethasone regulation of the HP promoter occurred in the apparent absence of a steroid receptor consensus binding sequence. We examined the role of glucocorticoid receptor by cotransfection experiments. HepG2 cells are known to have low

Fig. 3. Comparison of the 5'-flanking regions of HP genes. The sequence of the 5'-flanking region of the M. domesticus (C57BL/10) gene was determined from the isolated genomic clones and those of the M. caroli and M. saxicola from polymerase chain reaction products (see "Materials and Methods"). The sequence of the upstream primer is double-underlined. The recognition sites for transcription factor are indicated. The arrows mark the end points of regions used in CAT reporter gene constructs (see Table III).

Fig. 4. Regulated expression of HP-CAT constructs in HepG2 and H-35 cells. HepG2 (A) and H-35 (B) cells were transfected with a plasmid DNA mixture composed of M. domesticus HP (-227 to +20); OCT (17 µg/ml) and pIE-MUP. Subcultures of the transfected cells were treated for 24 h with the indicated factors. CAT activity in equal aliquots of cell extracts were determined and in A expressed as specific activity relative to the transfection marker MUP (number above thin layer pattern). The cytokine regulation of the endogenous haptoglobin in each culture was determined by rocket immunoelectrophoresis of 50-µl medium aliquots.
Table II
Regulated expression of mouse HP-CAT gene constructs in H-35 cells

H-35 cells were transfected with plasmid DNA mixtures composed of pHP-237 to +20-OCT (18 µg/ml) of M. domesticus, M. caroli, or M. saxicola and pIE-MUP. The specific CAT activity for each culture was normalized to the untreated control (-1.0) in the particular experimental series. Mean values ± S.D. (or range in case of duplicates) are shown.

| Treatment           | Relative expression |
|---------------------|---------------------|
|                      | M. domesticus | M. caroli | M. saxicola |
| None                | 1.0           | 1.0       | 1.0         |
| Dexamethasone       | (0.8 ± 0.2)²  | (0.9 ± 0.2)² | (1.3 ± 0.3)² |
| IL-1                | 1.3 ± 0.5 (6)  | 2.3 ± 0.7 (6) | 1.8 ± 0.6 (3) |
| IL-1 + dexamethasone| 62 ± 29 (5)   | 88 ± 7 (3) | 44          |
| IL-6                | 2.9 ± 0.4 (4)  | 7.0 ± 4.0 (4) | 2.4 ± 0.4 (2) |
| IL-6 + dexamethasone| 105 ± 78 (4)  | 165 ± 50 (3) | 48          |
| IL-1 + IL-6         | 2.1 ± 0.9 (2) | 4.7 ± 0.8 (2) | 4           |
| IL-1 + IL-6 + dexamethasone| 83 ± 8 (2)| 239 ± 7 (2) | 176         |

* Specific CAT activity (% conversion/h x ng MUP).

Table III
Regulatory activities of 5'-flanking regions of the M. domesticus HP gene

H-35 cells were transfected with the indicated HP CAT constructs (17 µg/ml) and pIE-MUP. Subcultures of the transfected cells were treated with Dexamethasone or IL-6, and the specific CAT activities of each culture were normalized to the activity of the untreated cell cultures (-1.0). Mean values ± S.D. (or range for duplicates) are shown.

| HP construct | Relative expression |
|--------------|---------------------|
|              | Dexamethasone | IL-6 |
| -1300 to +20 OCT | 16 ± 5 (2) | 2.8 ± 0.6 (2) |
| -237 to +20 OCT  | 76 ± 36 (8) | 2.9 ± 0.4 (4) |
| -184 to +20 OCT  | 7.2 ± 3.1 (3) | 2.5 ± 0.7 (3) |
| -112 to +20 OCT  | 3.0 ± 1.3 (3) | 2.1 ± 0.3 (3) |
| -237 to -106 CT  | 5.2 ± 2.5 (3) | 2.3 ± 1.1 (3) |
| -237 to -185 CT  | 1.9  | 1.9 |
| 3×(-237 to -185) CT | 1.6  | 0.6 |

 amounts of glucocorticoid receptors; thus, these cells are able to support only a limited steroid response unless supplemented with additional glucocorticoid receptor (45). As seen in Table IV, both the A-B-C region (-237 to -106) and the -112 to +20 region of M. domesticus produced a roughly equal magnitude of dexamethasone stimulation that was dependent upon co-expressed glucocorticoid receptor (GR). Subcultures were treated with medium alone (Control) or medium containing dexamethasone or IL-6. The specific activity in each culture was determined and expressed relative to the control of the "No addition" transfections (defined as 1.0). Mean values ± S.D. of duplicate experiments and mean ± S.D. for experiments with N > 3 are shown.

Table IV
Effect of glucocorticoid receptor on mouse HP gene promoters

HepG2 cells were transfected with plasmid DNA mixtures consisting of the indicated HP-CAT constructs of M. domesticus, M. saxicola, and M. caroli (5 µg/ml), pIE-MUP (2 µg/ml), and expression vectors for the glucocorticoid receptor (5 µg/ml each). The specific CAT activities in the subcultures were determined. Values for each treatment are expressed relative to the activity of the same constructs transfected with pIE-MUP and pIE-SVGR only; the latter, defined as 1.0, were taken from Table IV (samples marked GR). Mean values of duplicate experiments are shown.

| Construct | Relative expression |
|-----------|---------------------|
| M. domesticus |                      |
| -237 to +20 |                      |
| No addition | 1.0                  |
| GR | 0.5 ± 0.0 (4) |
| -112 to +20 |                      |
| No addition | 1.0                  |
| GR | 1                   |
| -237 to -105 |                     |
| No addition | 1.0                  |
| GR | 1                   |
| M. saxicola |                      |
| -237 to +20 |                      |
| No addition | 1.0                  |
| GR | 1                   |
| M. caroli |                      |
| -237 to +20 |                      |
| No addition | 1.0                  |
| GR | 1                   |

Table V
Transactivation of mouse HP gene promoters by transcription factor

HepG2 cells were transfected with plasmid DNA mixture containing the indicated HP-CAT constructs pIE-MUP, pIE-SVGR, and expression vectors for the indicated transcription factors (5 µg/ml each). The specific CAT activities in the subcultures were determined. Values for each treatment are expressed relative to the activity of the same constructs transfected with pIE-MUP and pIE-SVGR only; the latter, defined as 1.0, were taken from Table IV (samples marked GR). Mean values of duplicate experiments are shown.

| Construct | Relative expression |
|-----------|---------------------|
| M. domesticus |                      |
| -237 to +20 |                      |
| C/EBPβ | 8                   |
| PEA3 | 16                  |
| PEA3 + C/EBPβ | 39               |
| Fli-1 | 5                   |
| PU.1 | 0.3                 |
| Ets-2 | 2                   |
| -112 to +20 |                      |
| C/EBPβ | 10                  |
| PEA3 | 18                  |
| -237 to -105 |                   |
| C/EBPβ | 4                   |
| PEA3 | 1                   |
| M. saxicola |                      |
| -237 to +20 |                      |
| C/EBPβ | 37                  |
| PEA3 | 22                  |
| M. caroli |                      |
| -237 to +20 |                      |
| C/EBPβ | 4                   |
| PEA3 | 1                   |
| Fli-1 | 2                   |

C/EBPβ and the glucocorticoid receptor.

To document C/EBPβ binding to the predicted recognition sites within the promoter, we performed nuclease protection analysis. Nuclear extracts of COS cells that were transfected with C/EBPβ expression vector served as a source of C/EBPβ.
Fig. 5. Identification of C/EBPβ binding sites in HP gene promoters. A, binding of CCS cell-derived C/EBPβ to M. domesticus promoter. Nuclear proteins (0, 5, and 10 μg) from control COS cells (-C/EBPβ) and COS cells transfected with pCD-C/EBPβ (+C/EBPβ) were interacted with the M. domesticus HP promoter (-237 to +20) (end-labeled at either strand). The relative positions of the DNase I protected regions are indicated.
Within the −239 to +20 region of the *M. domesticus* HP gene, the two C/EBPβ binding sites at position A (−213 to −198) and C (−137 to −123) were observed (Fig. 5A). No obvious C/EBPβ binding was detectable within the proximal 112 bp of the promoter sequence, even though this sequence was a target of binding. The migration of co-electrophoresed size marker (length in nucleotides) is shown.

The Possible Role of Ets-related Factors in HP Gene Regulation—The promoter regions of the HP genes of *M. domesticus* and *M. saxicola*, but not *M. caroli*, contain a potential PEA3 recognition site (Fig. 3). PEA3, which is a member of the ets gene family, is expressed in adult mouse liver (Fig. 6). Although we failed to detect PEA3 in nuclear extracts of hepatoma cells by Western blotting using monoclonal antibodies against PEA3 (33), the physical presence of the PEA3 protein could conceivably influence expression of transfected HP gene constructs. To demonstrate that PEA3 interacts with predicted binding sites in HP promoters, a DNase I digestion pattern and location of protected region is shown.

We tested the effects of PEA3 on expression of HP CAT constructs in HepG2 cells by co-transfection (Fig. 8). A dose-dependent trans-activation was observed with the 237-bp and 112-bp *M. domesticus* HP CAT constructs. PEA3 in combination with C/EBPβ and glucocorticoid receptor showed the following effects (Table V): 1) PEA3 produced a significant trans-activation of all those constructs which contained the predicted PEA3 binding sites (i.e. those from *M. domesticus* and *M. saxicola*), but not of the HP CAT construct that lacked an obvious PEA3 consensus sequence (i.e. that from *M. caroli*); 2) PEA3...
ets-related genes were active in hepatic cells, we determined to gene by hormones in the hepatoma cells. In light of the fact that neutralized the IL-6 response and negatively interfered with drastically inhibited expression of the marker plasmid PIE-MUP. M. 

tasures were treated for 24 h with and without the action of the activated glucocorticoid receptor; and 3) PEA3 cooperated with C/EBPβ in transactivation. The mRNA analysis (Fig. 6) indicated that fetal and adult liver as well as hepatoma cells express mRNAs encoding members of the ets gene family. Messages for Fli-1 (3.7 kb) and Ets-2 (3.2 kb), but not for PU.1, were detected in liver and in HP gene-expressing hepatoma cell lines. The expression of ets family members was not appreciably influenced by an acute phase reaction in tissue culture (Fig. 2) truly reflects events in vivo, nor was it coordinately regulated with the HP gene by hormones in the hepatoma cells. In light of the fact that ets-related genes were active in hepatic cells, we determined to what extent Fli-1, Ets-2, and PU.1 could mimic the action of PEA3 on HP gene promoters (Table V). The results indicated that these three Ets-related factors exerted a much lower stimulatory or inhibitory activity than PEA3. Taken in total, these studies suggest a regulatory role in HP gene expression for members of the ets gene family.

**DISCUSSION**

The major conclusions of the present study are: 1) HP is a major acute phase protein in M. domesticus, M. caroli, and M. saxicola (Fig. 1; Table I); 2) the magnitude of stimulation in vivo is approximately 30-fold, which is 5 times higher than in rat (46); 3) the 5'-flanking region (Fig. 5) contains extended segments of homology among rodents and humans; 4) the 5'-flanking regions of the mouse HP genes from each of the three species mediates strong dexamethasone responses and minor IL-6 stimulation in transfected hepatoma cells (Fig. 4), accounting in part for the HP regulation seen in primary liver cells (Fig. 2); 5) a major response to IL-1 is observed only in M. caroli and M. saxicola; and 6) during evolution of the Mus genus, a purine-rich sequence was introduced into the HP gene promoters of M. domesticus and M. saxicola, giving rise to a PEA3 binding site that is potentially regulated by members of the Ets transcription factor family.

The three mouse HP gene promoters are distinguished from those of the rat and human by their low cytokine response and their prominent stimulation by dexamethasone (12, 25). (The glucocorticoid regulation of the human HP gene has yet to be demonstrated.) Of most interest is the finding that differences in HP gene regulation exist among the three mouse species. A small yet significant response to IL-1 occurs in M. caroli and M. saxicola, but not in M. domesticus (Fig. 2, Table II). In addition, a PEA3 response element exists in M. domesticus and M. saxicola, but not in M. caroli (Table IV). These features of HP regulation are summarized in Table VI.

The major acute phase protein gene expression and their response to acute inflammation are evolutionarily conserved (7). Hence, it might be assumed that the mode of regulation of these genes in the various species is similar, if not identical. Our results show that while the hepatic expression of the HP gene is similarly increased during the inflammatory reaction in each species, the relative activity of elements mediating the reaction appears rather variable. Results summarized above indicate that IL-1 contributes to the response in M. caroli and M. saxicola, while Ets-like proteins participate in M. domesticus and M. saxicola (Table VI). The previous observation that rat and human HP genes do not display identical cytokine response patterns (12, 17) has been simply accepted as a logical consequence of evolution that separated these species roughly 100 million years ago. Observing equally prominent differences in regulation among rodent species, which separated less than 20 million years ago, is unexpected. Our findings raise two critical issues that may be important in evaluating acute phase gene regulation: 1) what are the physiological implications of distinct cytokine requirements for acute phase plasma protein regulation? and 2) what are the molecular genetic mechanisms underlying the differences in the regulatory phenotypes?

Acute phase reactants exert a broad array of critical functions related to homeostasis, immune regulation, and tissue repair (47, 48). Therefore, the ability to appropriately regulate expression of acute phase reactants, including HP, is considered to be a process that is essential for survival of the organism in its natural habitat. The mode of acute phase protein stimulation is optimally adapted to the body's program. Several kinds of physical insult, including tissue damage, infection, inflammation, or immune reactions, activate the response. The existing data imply that inflammatory reactions requiring the induction of HP are primarily mediated by IL-6-type cytokines in human and M. domesticus; in contrast, both IL-1- and IL-6-type cytokines mediate HP induction in rat, M. caroli, and M. saxicola. This conclusion is valid only if the type of regulation defined in tissue culture (Fig. 2) truly reflects events in vivo and that a maximal level of expression has to be attained in order to meet the body's demand of HP or any other acute phase protein. An alternative possibility is that the magnitude of HP observed under experimental conditions in vitro or in liver in vivo is in excess of what is required to satisfy the "normal" need of the organism.

**To define the physiologically relevant mechanisms for regulation of the HP gene, knowledge about the acute phase-mediated changes in humoral factors is needed. Indeed, analysis of the response in human or rodents has invariably documented a concerted increase in IL-1- and IL-6-type cytokines along with**
glucocorticoids and other endocrine hormones (49). Considering the redundancy of hormone information, the precise contribution of a given factor to the overall regulation in vivo may be difficult to delineate. The relevance of specific cytokines, in particular IL-6, on HP gene regulation in mice will be gained by the analysis of recently established ILS-deficient mice.3

Regulation of the HP gene in liver is not only dependent upon the profile of factors activated by inflammatory processes, but also upon the composition and arrangement of cis-acting regulatory sequences within the gene. Earlier comparisons of rat and human HP genes has emphasized the similarity in the IL-6 response elements (12, 17, 18, 25) and the role of the C/EBP isomers in controlling HP gene expression (19, 21, 23). The issue of glucocorticoid action on the HP gene has received little attention due to the absence of obvious glucocorticoid response element consensus sequences within the gene; in addition, the dexamethasone effect on rat and human HP gene is only apparent in the presence of IL-6 (12, 25).

The present study suggests a stimulatory role for glucocorticoids that is characteristic for mouse HP genes. Dexamethasone enhances expression of the chromosomal HP gene in liver (Table I) and in primary hepatocyte culture (Fig. 2), although the magnitude of this stimulation was far less than that of the transfected HP-CAT constructs (Tables II-IV). A potential explanation for such a discrepancy is that a cis-acting repressor element has been removed during subcloning of the HP gene elements. Alternatively, the cellular environment of immortalized hepatoma cells may be different from that of liver cells. The latter explanation deserves attention because of the data on expression of ets gene family members (Fig. 6). Moreover, mouse hepatoma Hepa-1 cells display a dexamethasone regulation of the endogenous HP gene (Fig. 6, Ref. 31) that is highly reminiscent of the regulation of transfected HP-CAT constructs.

The promoter analysis (Table II) indicates that at least two regions of the HP gene promoter are sensitive to the glucocorticoid receptor (−237 to −104 and −112 to +20); also, sequences upstream (−1200 to −238) exert an inhibitory effect. It is tempting to speculate that these elements are the ones which are also necessary for steroid response of the chromosomal genes in liver and in Hepa-1 cells. Functional characterizations of HP gene constructs, including a more detailed analysis of HP transgenes, must be done to gain further information on the mechanism underlying the steroid response.

The action of the glucocorticoid receptor on the acute phase genes has invariably been found to involve cooperativity with other factors (13, 45, 50–55). A positive function could be ascribed to C/EBP isomers, since removal of the C/EBP recognition sequence in the A element (−237 to −185) lowered the dexamethasone response by severalfold (Table III). Furthermore, co-expressed C/EBPβ enhanced dexamethasone stimulation in constructs containing the sequence from −237 to −105 (Table V). This would suggest that the transcription factor composition (including C/EBP isoforms) present in hepatoma cells and assembled on the transfected mouse HP gene elements cooperate with the glucocorticoid receptor in mediating induction by the steroid. The identity of the relevant participating factors, the location of the glucocorticoid receptor binding site, and the nature of the cooperative actions among factors are subjects of future studies.

The evolution of the Mus genus is accompanied by characteristic modifications in the HP gene promoter region (Fig. 3). The polypurine segment adjacent to the A-B-C region probably contributes to the regulatory phenotype of the HP gene in M. domesticus and M. saxicola. This sequence may affect productive communication between the transcription factors bound to the A-B-C region and other promoter elements. Alternatively, Ets-related transcription factors may bind to the polypurine sequence and modulate the activities of adjacent promoter elements. The second possibility is consistent with the observation that PEA3, and to a far lesser extent Ets-2 and Fli-1, have transactivating activity (Table IV), and that these factors are expressed in hepatic cells (Fig. 6). Expression of PEA3 in hepatoma cells is not surprising, since this factor has been implicated in oncogenesis (33). Transactivation of the HP gene by Ets-related proteins would explain in part activation of the HP gene during development, fine tuned expression during acute phase, and ectopic expression in neoplastic tissues (56, 57).

In this study, we attempted to associate changes in the structure of the HP gene promoter and alterations in regulation by cytokines and glucocorticoids. We cannot rule out that these species-specific sequences serve additional functions. These may include developmental activation of the HP gene and control of tissue-specific expression in liver and in extrahepatic sites. The molecular basis for these regulatory processes are still unknown in any of the studied species.

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Mouse Haptoglobin Gene Regulation
Mouse Haptoglobin Gene Regulation

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