Distinct Regulation of the Interleukin-1 and Interleukin-6 Response Elements of the Rat Haptoglobin Gene in Rat and Human Hepatoma Cells

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The transcription rate of the haptoglobin (Hp) gene is stimulated by interleukin-1 (IL-1), IL-6, and dexamethasone in rat hepatoma (H-35) cells. To identify the *cis*-acting regulatory elements responsive to these hormones, various lengths of 5' Hp gene-flanking regions, including the promoter, were inserted into chloramphenicol acetyltransferase gene expression vectors and transiently introduced into H-35 cells. The first 4 kb of 5' region mediated a severalfold increase in expression after treatment with IL-6 and dexamethasone. No response to IL-1 was detectable. When, however, upstream sequences were deleted to position –165 relative to the transcription start site, a significant stimulation by IL-1 was gained without appreciably affecting the IL-6 response. With the apparent removal of an inhibitory sequence, the promoter-proximal 165-bp region also displayed a severalfold enhanced response to the combination of dexamethasone, IL-1, and IL-6. The sequence from –165 to –147, termed the A-element, was found to be crucial for all hormone regulatory functions. Two copies of the A-element linked to a heterologous promoter responded to the three hormones, but to a lesser degree than in the Hp gene promoter context. The regulatory elements of the rat Hp gene were similarly active in human hepatoma cells. Optimal regulation by IL-6 in HepG2 cells was, however, independent of the A-element. The A-element functioned in these cells exclusively as an IL-1 response sequence. The results suggest that genomic sequences upstream of the rat Hp gene suppress the regulation by specific cytokines more prominently in transient expression assays than in the normal chromosomal context. Moreover, the functional comparison indicated that specific regulatory regions of the rat Hp gene do not function identically in different hepatic cell types.

Haptoglobin (Hp) belongs to the group of plasma proteins whose synthesis is increased severalfold as a consequence of systemic tissue injury (30). Potential mediators for this acute-phase liver response have been identified in liver cell cultures which have retained an overall pattern of plasma protein regulation as observed *in vivo* (4, 18). Interleukin-1 (IL-1), tumor necrosis factor α , IL-6, leukemia inhibitory factor (LIF), and glucocorticoids, alone or in various combinations, were found to stimulate Hp production in hepatic cells from rats, mice, and rabbits (9, 34, 47). In contrast, Hp expression in human liver cells is primarily stimulated by IL-6 and LIF (11, 41), and this action is synergistically enhanced by glucocorticoids (10, 13).

To understand the molecular mechanism of hormone action, the functional Hp gene from humans (12) and rats (37) has been cloned. The *cis*-acting regulatory element of the human gene which is responsive to IL-6 in transiently transfected Hep3B cells has been localized to the promoter region at position –180 to –50 relative to the transcription start site (43). This element consists of three separate, cooperatively interacting sequences, A, B, and C (42).

The rat Hp gene sequences, spanning from –6500 to +13000, have been similarly tested for regulatory elements by transient transfection into HepG2 cells (37; S. Marinkovic and H. Baumann, unpublished data). The results indicated the presence of one major regulatory element at position –146 to –56 which is responsive to IL-6, LIF, and dexa-

methasone (11, 37). This element appears to consist of two interacting regions which are similar in sequence to the two proximal regulatory regions B and C of the human gene (42). The rat homolog to the human region A seemed, however, to be inactive.

Although the functional analysis of the rat Hp gene sequences in HepG2 cells indicated the presence of an element responsive to IL-6 and glucocorticoids, it did not reveal any evidence for an IL-1 response element (37). Considering that the transfected Hp gene, like the endogenous human Hp gene, was not affected by IL-1, the question arose as to whether the Hp gene expression was indeed regulated at the transcription level in rat hepatic cells by IL-1 and, if so, whether the human hepatoma cells were deficient in *trans*-acting factors which would be required for IL-1 stimulation of the rat gene. A most suitable experimental cell system to address this question appeared to be a subline of Reuber H-35 rat hepatoma cells, which respond to the inflammatory cytokines by increasing the production of most acute-phase plasma proteins, including Hp, to the level observed for an acute-phase liver (9). In addition, transfection of plasmid DNA into these cells has been improved, allowing transient functional analysis of gene elements with the same sensitivity as obtained previously with human hepatoma cells. Using these cells, we observed that IL-1 stimulated Hp gene transcription more strongly than IL-6. A potent IL-1-responsive element could be identified in the Hp gene promoter region after removal of an adjacent, previously unrecognized, inhibitory sequence. A functional comparison of the rat Hp gene elements in rat and human hepatoma cells revealed that IL-1 regulation in both cell

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types utilized the same sequences but that optimal IL-6 regulation in rat cells relied on an arrangement of regulatory sequences which differed from that which was active in human cells. The data presented in this work provide evidence that functional analyses of regulatory elements from acute-phase proteins might be potentially influenced by cell-specific differences in *trans*-acting factors. Moreover, regulatory sequences which have been integrated into an expression vector might not always be properly presented to the *trans*-acting factors for producing a hormone response as observed for the gene in its normal chromosomal environment.

MATERIALS AND METHODS

Cells and cytokines. In this study, we used a repeatedly subcloned line of the Reuber H-35 rat hepatoma cell, clone T-7-18 (9). H-35, HepG2 (28), and Hep3B-2 cells (15) were cultured as described previously.

Bacteria-derived human recombinant IL-1 α was generously provided by S. Dower, Immunex Corp., Seattle, Wash. Human recombinant IL-6 was obtained from conditioned medium of COS cells transfected with the IL-6 expression vector pXM309 (52) and human recombinant LIF from COS cells transfected with the LIF expression vector (40) (both vectors generously provided by G. Wong, Genetic Institute, Cambridge, Mass.). Hepatic activity of IL-6 and LIF was determined by the stimulation of fibrinogen and Hp synthesis in HepG2 cells and the activity of IL-1 by the stimulation of complement C3 in H-35 cells (10, 11, 38). Unless noted otherwise, the following standard hormone treatments were used: serum-free minimal essential medium alone or with 500 U of IL-1 per ml, 250 U of IL-6 per ml, 10 U of LIF per ml or 1 μ M dexamethasone.

Analysis of cell response. Nuclei were prepared from individual monolayers in 10-cm-diameter culture dishes by an abbreviated procedure of Almendral et al. (2). Briefly, cells were scraped off the culture dish and washed once in phosphate-buffered saline and then lysed for 5 min in 1 ml of a solution consisting of 20 mM Tris (pH 7.4), 10 mM NaCl, 2 mM MgCl₂, 0.5% Nonidet P-40, and 20 U of RNasin per ml. The nuclei were recovered by centrifugation for 10 s at top speed of the microcentrifuge (13,000 $\times$ g) and washed once with 1 ml of storage buffer (40% glycerol, 5 mM MgCl₂, 50 mM Tris [pH 7.5], 0.1 mM EDTA). Cytoplasmic RNA was isolated from supernatant lysates (14), and the relative amounts of mRNAs for acute-phase plasma proteins were determined by dot blot hybridization to ³²P-labeled cDNAs. The transcription run-on reaction and isolation of nuclear RNA followed the procedure of Lamers et al. (31). Nuclei derived from one 10-cm-diameter dish yielded between 2 $\times$ 10⁷ to 5 $\times$ 10⁷ cpm of ³²P-labeled transcripts. Equal amounts of radioactivity were hybridized to nitrocellulose strips carrying slot-blotted denatured cDNAs encoding rat Hp, thiostatin, α -fibrinogen, hemopexin (6), α_2 -macroglobulin (22), or the genes for rat α_1 -acid glycoprotein (8), chloramphenicol acetyltransferase (CAT), and human triosephosphate isomerase (36). The change in the amount of secreted plasma proteins was measured by rocket immunoelectrophoresis of equal samples of medium.

Plasmid constructs. Various lengths of Hp gene 5'-flanking region, including its promoter, were inserted into pSVOCAT and have been tested for cytokine regulation in HepG2 as previously described (37). In addition, subfragments of the Hp gene promoter region were inserted into the polylinker 5' to the adenovirus major late promoter linked to the CAT

gene in pUC18 (pCT; a gift of D. Grayson, Georgetown Medical School). The double-stranded oligonucleotide representing the IL-1 regulatory element (A-element) at position -165 to -147 (CAAGTATGAAGCAAGAGCT) and the human equivalent (CAAGTGTGAAGCAAGAGCT) were synthesized at the Roswell Park Cancer Institute biopolymer facility and inserted as *Eco*RI-*Sst*I fragments into pCT. Two copies of these sequences, separated by an 88-bp *Pvu*II-*Eco*RI fragment of pUC13, were similarly inserted in either orientation into pCT. For comparison of cytokine-specific regulation, we also used plasmid p Δ 14CRP-CAT, which contains 565 bp of flanking region from the human C-reactive protein gene in pEMBL131+ (3) (generously provided by G. Cliberto, EMBL), and plasmid p(4 $\times$ AB)-SV-CAT, which contains four copies of the 62-bp IL-1 response sequence of the rat α_1 -acid glycoprotein gene upstream of the enhancer-less simian virus 40 promoter in pSV-CAT (51).

Cell transfection. HepG2 and Hep3B cells in 10-cm-diameter culture dishes were transfected with 20 μ g of plasmid DNA as a calcium phosphate precipitate (24). For all transfections of HepG2 cells, 2 μ g of pRSVGR plasmid DNA was included in order to complement the glucocorticoid receptor deficiency of these cells (39). H-35 cells were transfected with the same amounts of DNA but as a DEAE-dextran complex (33). After overnight recovery, the cells were released by trypsin and equal portions were plated into 8 to 10 (10-cm²) wells of 6-well culture plates. Twenty-four hours later, the cells were treated for an additional 24 h in 1 ml of serum-free medium without and with cytokines or dexamethasone. In those experiments involving 12-*O*-tetradecanoylphorbol-13-acetate (TPA), the treatment period was limited to 8 h (7). The activity of CAT activity in cell extracts was determined and related to the amount of protein present (23). The specific CAT activities derived for the treated cells were expressed as ratio to the control cultures (fold stimulation).

RESULTS

Transcriptional regulation of Hp gene. We have previously reported that the concentration of cytoplasmic Hp mRNA is increased after treatment of H-35 cells with various cytokines and dexamethasone (5, 9). To assess the contribution of transcriptional changes to the overall regulation of Hp expression, the effect of individual hormones and combination of these on Hp gene transcription was determined (Fig. 1A) and related to the level of cytoplasmic Hp mRNA and the amount of Hp secreted by the same cells (Fig. 1B). To illustrate the hormone specificity of Hp regulation in the context of the hepatic acute-phase reaction, the responses of other, representative acute-phase protein genes are reproduced. The housekeeping enzyme, triosephosphate isomerase, served as a reference gene whose transcription was not affected by the hormone treatments.

The Hp gene, like the other acute-phase plasma protein genes analyzed in Fig. 1, showed changes in the transcription rate which generally agreed with the increases in the mRNA concentration and synthesized proteins. However, minor quantitative discrepancies also appeared to exist. A 12-h treatment with IL-1 prominently stimulated the rate of Hp gene transcription and this response was enhanced twofold by dexamethasone (Fig. 1A). In contrast, IL-6 alone or in combination with dexamethasone elicited only minimal increases. The levels of Hp mRNA attained after either cytokine treatment were, however, surprisingly similar (Fig. 1B), suggesting that the cytokines might affect some post-

FIG. 1. Regulated expression of Hp in H-35 cells. (A and B) Hormone specificity. Confluent monolayers of H-35 cells in 10-cm-diameter dishes were treated for 12 h with serum-free medium containing the indicated factors. Dex, Dexamethasone. (A) Nuclei were prepared and used for run-on reaction. Labeled RNAs (3×10^7 cpm each) were hybridized to slot-blotted DNA encoding α_1 -acid glycoprotein (AGP), thioistatin (TST), Hp (HP), α -fibrinogen (α FB), hemopexin (HPX), and triosephosphate isomerase (TPI), and pBR322 (pBR). The autoradiogram after a 10-day exposure is shown. (B) Serially diluted cytoplasmic RNAs were probed with 32 P-labeled cDNAs encoding α_1 -acid glycoprotein, thioistatin, and Hp. Seventy microliters of the culture medium was analyzed for the same proteins by rocket immunoelectrophoresis. (C) Time course of transcriptional activation. H-35 cells were treated for the indicated length of time with a mixture of either IL-6 and dexamethasone or IL-1, IL-6, and dexamethasone, and transcription rates were determined by nuclear run-on reaction. α_2 MG, α_2 macroglobulin. (D) Cycloheximide-inhibited cytokine regulation. H-35 cell monolayers were pretreated for 15 min with serum-free medium alone or containing 10 μ g of cycloheximide (CHX) per ml, before adding IL-1, IL-6, and dexamethasone. After 4 h, the levels of gene transcription and cytoplasmic RNA were determined.

transcriptional processes in addition to transcription, i.e., mRNA processing, transport, or cytoplasmic stability. Combination of IL-1 and IL-6 resulted in an additive to synergistic action on Hp gene transcription and mRNA accumulation.

The hormone-specific effect, as seen after 12-h treatment (Fig. 1A), was not attributable to differences in kinetics of gene activation. Various combinations of the hormones yielded essentially the same time course (representative example in Fig. 1C). A slight elevation of the transcription rate was noted after 1-h treatment, and a significant increase was achieved after 6 h. The enhanced transcription rate persisted, if not further increased, during the following 18 h. The temporal changes of Hp gene transcription paralleled that of the other acute-phase plasma protein, illustrating the coordinate nature of the hepatic response to the inflammatory hormones.

Although a remarkably similar cytokine specificity for the regulation of Hp has been observed in hepatic cells from various species (3, 10, 13, 34, 47), cell-specific differences in cytokine action exist. One difference is noteworthy in view of the present comparison of Hp regulation in rat and human hepatoma cells. The stimulation of the Hp gene or any other acute-phase plasma protein gene by both IL-1 and IL-6 is strongly dependent on ongoing protein synthesis in H-35 cells (Fig. 1D), but not in HepG2 cells (6; data not shown). H-35 cells whose protein synthesis is inhibited by cycloheximide showed only a brief, transient stimulation of Hp gene expression that is reflected in a reduced Hp mRNA accumulation and a return to basal-level transcription after 4 h of cytokine treatment.

From the above results we reached the following conclusions: (i) the rat Hp gene must have regulatory elements responsive to IL-1, IL-6, and dexamethasone, (ii) the transcriptional activity of the Hp gene can be maintained in a stimulated state for hours, and (iii) the cytokine signaling to the Hp gene elements in H-35 cells involves one (or more) labile protein(s) present at a low concentration. Since we wished to understand the molecular mechanisms of Hp gene control, we have to establish whether the cloned rat Hp gene sequence contains the structural information for IL-1 regulation and whether cooperativity between IL-1 and IL-6 elements would explain the additive to synergistic action of the two cytokines.

Regulation of transfected rat Hp gene sequences in H-35 cells. The rat Hp gene promoter and adjacent 5'-flanking region have previously been characterized for the IL-6 and dexamethasone response element in HepG2 cells (37). To corroborate those findings in a rat system, we introduced into H-35 cells those gene constructs which were found to be responsive to the hormones. The transiently transfected cells were then treated with various combinations of hormones to assess the functionality of the Hp gene sequences. The length of treatment was generally 24 h, a time period sufficient for attaining high transcription rates for the endogenous Hp gene (Fig. 1C) as well as the transfected plasmid (Fig. 2B). The cytokine regulation of the two most distinct constructs along with the endogenous Hp gene is illustrated by the results of a representative experiment in Fig. 2A. The numerical values for the stimulation of various constructs are summarized in Figure 3.

Plasmids containing either 4 kb or only 400 bp of immediate 5'-flanking sequence of the Hp gene responded to dexamethasone and IL-6 by a 5- to 8-fold increased expression (constructs 1 and 2 [Fig. 3]). The action of the two hormones appeared to be additive. IL-1, either alone or in combination

with IL-6 or dexamethasone, failed to exert any stimulatory effect. The regulation of these plasmids was qualitatively identical to that in HepG2 cells (Fig. 2C) (37); however, the magnitude of dexamethasone response was 2- to 3-fold higher on average in H-35 cells. The cytokine regulation was not significantly improved by adding the first exon region up to position +26 that included the sequence TCTGGAAA (+7 to +14), a motif associated with IL-6 response elements of other acute-phase plasma protein genes (19, 25, 29) (constructs 7 and 8).

Deletion of Hp sequences 5' to position -165 yielded a promoter (construct 3) which, although less responsive to dexamethasone, was now strongly stimulated by IL-1 and synergistically by IL-1 and IL-6. The transcriptional activation of that construct in transfected cells by IL-1, IL-6, and dexamethasone was comparable with the endogenous Hp gene (Fig. 2B). The hormone responsiveness of the Hp gene element was severely reduced by further deletion of 19 bp of 5'-flanking sequence (construct 5) and was completely lost after truncation to position -87 (construct 6). The relative low activity of the former construct was unexpected, because a functional test in HepG2 cells had indicated that its IL-6 response was equal to those of constructs carrying extended 5' Hp gene regions, e.g., constructs 1 and 2 (37). To rule out the possibility that the peculiar regulatory properties of the Hp constructs in H-35 cells was attributable to immediately adjacent pSVOCAT vector sequences brought in juxtaposition with the Hp gene sequences, the promoter segments of constructs 2, 3, 5, and 6 were also inserted into promoterless pCT (data not shown). The values for fold stimulation obtained for those constructs were virtually identical to ones presented in Fig. 3. From these data, we reached the following conclusions: (i) cloned Hp gene sequences contained all the elements needed to achieve appropriate hormone regulation, (ii) sequences upstream of -165 prevented functionality of the IL-1 regulatory element, and (iii) optimal IL-1 and IL-6 regulation of the Hp gene promoter in H-35 cells requires the sequence from -165 to -147.

The sequence from -165 to -147 is the homolog to the human Hp gene segment A containing a crucial constituent of the IL-6 regulatory element (42). The rat sequence differs from the human by a single base at position -160, an adenosine instead of guanine. That difference was assumed to be causal for reduced IL-6 responsiveness of the rat element (37). By changing that nucleotide to the human equivalent, the IL-6 responsiveness of the resulting construct improved not only in HepG2 cells (37) but also in H-35 cells (construct 4 [Fig. 3]). The mutation had a twofold enhancing effect on IL-6 response, but that improvement was not proportionally manifested when treated in addition with IL-1 and dexamethasone.

The cytokine response element active in H-35 cells was further defined by testing subregions of the Hp gene sequence in combination with the adenovirus major late promoter in pCT. The combination of the promoter flanking region from -165 to -56 with the viral promoter (construct 9) resulted in a plasmid which was still responsive, albeit to a lesser extent to all three hormones. Moreover, no significant synergy between the hormones was detectable (compare constructs 9 and 3). Apparently, the Hp gene promoter sequences are necessary for gaining optimal functionality of the cytokine element, an observation that has been made previously for the human Hp gene (42, 43). The importance of the 5' 19-bp sequence was again demonstrated by the loss of regulation upon deletion (construct 10). A single copy of

FIG. 2. Regulation of transiently transfected Hp-CAT constructs in H-35 (A and B) and HepG2 (C) cells. (A and C) One monolayer culture of each cell type was transfected with plasmid pHp(400)-CAT (contains Hp sequence from -396 to -2 ; construct 2 [Fig. 3]) or pHp(170mut)-CAT (containing Hp sequence from -165 to -2 with an A-to-G change at -160 ; construct 4 [Fig. 3]). The transfected cells were divided into 8 to 10 separate wells and treated with the indicated factors for 24 h. The CAT activity and the amounts of plasma protein secreted by each culture were compared. (B) H-35 cells in 15-cm-diameter dishes were transfected with plasmid pHp(170)-CAT (contains Hp sequences from -165 to -2 , construct 3 [Fig. 3]). After the recovery period, the culture was released by trypsin and divided equally into two 10-cm-diameter dishes. Twenty-four hours later, the cells were treated either without (left lane) or with IL-1, IL-6, and dexamethasone (right lane) for additional 24 h. Transcription and cytoplasmic mRNA level were determined as described in the legend to Fig. 1. Abbreviations: Dex, dexamethasone; HP, Hp; AGP, α_1 -acid glycoprotein.

that 19-bp sequence did not show convincing regulatory functions (construct 11). Two copies of that sequence, separated by an inactive 88-bp pUC13 sequence (to avoid the possibility of an unfavorable conformation), yielded an element which displayed a response pattern that was surprisingly similar to that obtained with the entire region from -165 to -56 (compare constructs 9 and 12). Two copies of the same 19-bp fragment, but representing the human equivalent (construct 13), gave a comparable regulation except that the IL-6 response was between one- to two-fold elevated. These results indicate that the Hp regulatory element is composed of separable, cooperatively interacting sequences and that the qualitative information for regulation of Hp gene in rat cells could be reproduced by duplication of a 19-bp sequence. In accordance with the terminology established for the human gene (42), this 19-bp sequence is termed the A-element.

Function of the A-element in HepG2 cells. Because the plasmid pHp(170mut)-CAT (construct 4 [Fig. 3]) showed a striking multihormone responsiveness, its activity in H-35 cells (Fig. 2A) was compared with that in HepG2 cells (Fig. 2C). Indeed, this construct in HepG2 cells, as in H-35 cells, was responsive not only to IL-6 but also to IL-1. IL-1 and

IL-6 acted additively, but not synergistically with dexamethasone. The equivalent, wild-type sequence (construct 3) yielded in HepG2 cells a similar magnitude of IL-1 stimulation (data not shown). On the basis of the previous data (37) and the results presented in Fig. 3, the maximal IL-6 regulation of the rat Hp gene appears to demand a different composition of 5'-flanking regions, depending upon whether the assay was carried out in HepG2 or H-35 cells. The most notable feature was that the rat A-element appeared to be unnecessary for IL-6 response in the human hepatoma cells, but essential in rat cells. The data of Fig. 2C suggest that the A-element might act nevertheless as an IL-1 response element in HepG2 cells. Transfection of the two-copy A-element construct (construct 12 [Fig. 3]) into both cell types revealed pointedly the cell-specific difference (Fig. 4). In HepG2 cells, there was no detectable regulation by IL-6 or dexamethasone but a ninefold stimulation by IL-1. Addition of IL-6 to IL-1 had no enhancing effect. In contrast, the same construct in H-35 cells mediated a regulation which was sensitive to all three hormones with prominent synergy between dexamethasone and IL-1 or IL-6. The functional tests in HepG2 cells therefore suggests that the A-element contributes solely to the IL-1 response and that when this

Construct		Fold Stimulation of CAT Activity						
		Dex	IL-1	IL-1+Dex	IL-6	IL-6+Dex	IL-1+IL-6	IL-1+IL-6+Dex
1		7.5	0.7	3.8	7.7	16.8	5.0	14.0
2		6.5	1.1	8.5	4.8	7.9	2.3	7.2
3		2.4	2.6	38.5	5.6	20.4	25.6	105.2
4		5.2	5.0	31.0	9.0	37.1	50.4	123.5
5		2.0	1.0	1.7	1.7	2.6	1.5	3.6
6		1.2	0.7	1.0	1.1	1.2	0.9	0.9
7		4.7	0.6		3.3	9.2	1.8	
8		1.3	1.1		1.3	2.5	1.8	
9		1.9	1.9	7.4	2.8	8.1	2.8	6.8
10		1.1	1.0	1.3	1.3	1.3	1.1	1.1
11		1.3	1.2	1.6	1.2	1.6	1.2	1.6
12		1.6	1.8	5.7	2.3	6.9	2.2	7.2
13		1.8	1.8	6.2	4.1	9.3	5.0	10.2
14		1.1	1.0	1.0	1.2	1.3	0.9	1.0

FIG. 3. Cytokine-specific response of the regulatory elements of the Hp gene in H-35 cells. CAT plasmid constructs containing the indicated Hp gene 5'-flanking regions in pSVOCAT (constructs 1 to 8) or in pCT (constructs 9 to 13) were transfected into H-35 cells and tested for regulated expression by dexamethasone (Dex), IL-1, IL-6, and alone and in various combinations. The fold stimulation relative to the untreated control was determined in each experimental series. The values represent means of two to five separate experiments.

element is linked to the 3' IL-6 response element, an additive cytokine stimulation of the gene expression is achieved.

Cytokine response of rat Hp gene elements is independent of activated protein kinase A or C. Several recent publications have suggested that intracellular signal transduction for IL-1 (49, 50, 53, and Discussion in reference 44) and IL-6 (17) involves, in part, protein kinase A or C pathways. Indeed, transfection of regulatory sequences from acute-phase protein genes into a variety of cells have shown, at least, a partial positive response to phorbol ester treatment (7, 16, 48, 51). Since the rat Hp promoter region contains IL-1 and IL-6 response elements, the probability exists that it might serve as target of either protein kinase signaling pathway. Indeed, involvement of a generic transcription controlling factor would conveniently explain the molecular basis for the striking coordination of acute-phase plasma protein gene expression (16, 48). However, transfection of constructs containing the most significant Hp gene elements into H-35 or HepG2 cells failed to reveal any significant regulation by cyclic AMP or TPA (Fig. 5A shows the result of one representative construct in H-35 cells). That the cells were capable of regulating acute-phase gene elements through TPA is documented by cells which had been transfected with a CAT plasmid containing four copies of the IL-1 response region (AB) of the distal regulatory element of the rat α_1 -acid glycoprotein gene (51) (Fig. 5B). We can conclude from this

result that cytokine signal communication to the Hp gene probably does not involve activated protein kinase A or C.

The promoter proximal region of the Hp gene contains all the regulatory information elements necessary for a type 1 acute-phase protein gene. The rat acute-phase plasma proteins can be grouped into two categories: type 1 proteins that require IL-1 for maximal expression and type 2 proteins that are either independent or inhibited by IL-1 (9). As apparent from Fig. 1A, Hp, like hemopexin and α_1 -acid glycoprotein, belongs to the first group and thiostatin, fibrinogen, and α_2 -macroglobulin belong to the second. In addition, LIF can stimulate the expression of acute-phase proteins, preferentially of type 2 (11). However, type 1 proteins, like Hp, are also stimulated by LIF, albeit to a minor extent (Fig. 1A). Optimally functional Hp-CAT plasmid (construct 3 [Fig. 3]) transfected into H-35 cells was stimulated two- to threefold by LIF, and this response seems to be additive with that of IL-6, IL-1, and dexamethasone (Fig. 2A). A test of the various constructs (Fig. 3) indicates that the DNA sequences necessary for LIF response coincided with those needed for IL-6 response (data not shown). Together with the data on IL-1, IL-6, and dexamethasone regulation (Fig. 3), we conclude that all *cis*-acting regulatory information which are characteristic for a type 1 acute-phase protein can be recovered within the first 165 bp of 5'-flanking region. The specific functionality of these elements, as defined in H-35 cells,

FIG. 4. Activity of the Hp A-element in HepG2 and H-35 cells. One culture each was transfected with plasmid construct 12 (Fig. 3). The cultures were subdivided and treated with hormones. The CAT activity in equivalent amounts of cell extract was determined, and the values for percent conversion of substrate to product are indicated at the top. Dex, Dexamethasone.

appears to be more general in nature, since the cytokine-specific action onto these elements can also be reproduced in HepG2 cells (Fig. 2C) and Hep3B cells (Fig. 5C). In the latter cell type, no stimulation by LIF was detectable, most likely due to the fact that these cells are nonresponsive to that factor (A. Mackiewicz and H. Baumann, unpublished data). Nevertheless, the Hp sequence showed a pattern of cytokine regulation that was a mirror image of that achieved with the elements from the C-reactive protein gene, the only molecularly characterized human type 1 acute-phase protein described so far (3, 20, 21, 32).

DISCUSSION

The regulation of Hp in H-35 cells (Fig. 1) shares common features with that of normal rat liver cells, which includes the following: (i) the qualitative response to cytokines and dexamethasone, and (ii) the kinetics and absolute level of mRNA accumulation (9). However, a substantial deviation from the normal liver phenotype is also noted in H-35 cells: the basal level expression of the Hp gene is much reduced. Both the similarities and differences have to be kept in mind when interpreting the regulation pattern of the chromosomal versus the transfected gene and when comparing acute-phase regulation in normal rat liver cells with cytokine stimulation in tissue culture hepatoma cells. Aside from these reservations, H-35 cells can nevertheless be considered as a most suitable test system for *cis*-acting cytokine- and glucocorticoid-response elements, because these cells must contain all the necessary components or signal-generating circuitry which determine the magnitude and correct temporal activation of specific genes.

When comparing the expression of the endogenous Hp gene with transfected Hp-CAT constructs, one notices the quantitative differences in hormone response as well as the qualitative similarity (Fig. 2A and B and 3). The most prominent difference is the sevenfold dexamethasone stimulation of the transfected plasmid DNA, while the endogenous Hp gene is only minimally to nondetectably affected. Moreover, the magnitude of IL-6-stimulated CAT expression seems to exceed the transcriptional activation of the endogenous gene (Fig. 1A). Several reasons might contrib-

FIG. 5. Functional comparison of the Hp gene element with those from other acute-phase plasma protein genes. Plasmid pHp(170)-CAT (construct 3 [Fig. 3]) (A) and pAGP (4xAB)-SV-CAT (B) was transfected into H-35 cells, and pCRP-CAT and pHp(170)-CAT were transfected into Hep3B cells (C). The H-35 subcultures were treated for 8 h with 0.1 mM 8-bromo-cyclic cAMP (cAMP), 0.15 μ M TPA, or IL-1 before analysis of the CAT activity. The treatment of Hep3B cells with the indicated hormones was carried out for 24 h.

ute to this apparently inconsistent control of episomal DNA: the transfected constructs may be more accessible to the regulatory factors, methylation differences might render the construct more active, the *cis*-acting elements within the plasmid context may be more favorably displayed to mobile DNA-binding proteins, or the plasmids may lack the appropriate interactions with specific nuclear matrix components for assuming the conformation present in the chromosome (45). The importance of the structural presentation of a DNA sequence is best illustrated for IL-1 regulation. Transcription of the endogenous Hp gene is strongly stimulated by IL-1 (Fig. 1A), but the transfected plasmid is not, unless the flanking sequences have been trimmed to position -165 (Fig. 3). Such a gain of cytokine function has been interpreted as being a consequence of deleting an inhibitory element (32). Considering that this supposedly negatively acting sequence is present in the genomic Hp gene, in which it fails to exercise inhibition, the inhibitory potential of that element must be suppressed in the chromosomal Hp gene, e.g., by a structural modification which was not realized in the cloned piece. Previously reported data (37) and the data shown in Fig. 3 indicate that the upstream sequence does not act as a general inhibitor of plasmid transcription because an effect on basal level expression or magnitude of IL-6 stimulation has not been detected. Although the IL-1 response, uncovered by the specific deletion, would adequately account for the regulation pattern of the Hp gene as well as explain our failure to detect an IL-1-responsive element elsewhere in the Hp gene from -6500 to +13000, its true function in the normal rat Hp gene still remains to be determined.

A quite surprising finding is the close interaction between the signaling pathways of IL-1, IL-6, and dexamethasone which act on Hp regulatory elements. A case in point is the CAT vector with the duplicated A-element (construct 12) in which the 19-bp sequence seems to be the target of three hormone signal pathways in H-35 cells (Fig. 4). An alternative possibility would be that the cytokines or dexamethasone function, in part, indirectly by activation of a common

transcription-controlling component, either by increasing the transcription of its gene or alternatively increasing transcription of the gene encoding an enzyme activity which modifies the transcriptional component. An indirect action would explain the dependence of that hormone response on *de novo* protein synthesis (Fig. 1D). The fact that the 19-bp sequence mediates only an IL-1 response in HepG2 cells (Fig. 4) would suggest that the A-element is primarily the target of the IL-1 signal transducing system. The stimulatory effect of IL-6 and dexamethasone in H-35 cells would thus be explained by an indirect action onto the hypothetical IL-1-dependent *trans*-acting factor. An involvement of a generic IL-1 sensitive factor can, however, not yet be proposed in view of the fact that the A-element lacks the basic consensus sequence requirement for an IL-1 response element as defined recently (1). Nevertheless, the A-element contains one sequence-similar component also found associated with the core IL-1 regulatory unit of α_1 -acid glycoprotein gene (51). Ongoing analysis of Hp element-binding proteins and potential transcription factors will provide more definitive information as to the number of interacting proteins and their identity to already established IL-1-responsive nuclear factors (1, 16, 48).

The second important conclusion of this paper is that the degree of hormone regulation of a given construct is not identical in different hepatic cell types. The inferior dexamethasone regulation of Hp gene constructs in HepG2 cells compared to H-35 cells is most likely tied to the relatively low glucocorticoid receptor levels (H. Baumann, unpublished data). Even when supplemented by cotransfection with pRSVGR, the magnitude of stimulation is still twofold less than in H-35 cells. A more intriguing situation seems to apply to the sequence requirements for reconstitution of a functional IL-6 regulatory element. The rat equivalents to the B and C of the human Hp gene elements are sufficient in HepG2 cells for gaining maximal IL-6 response (37), whereas in H-35 cells, the same combination was minimally active. Only with addition of the A-element was a prominent IL-6 regulation achieved (Fig. 3). The need for the latter element for full functionality in H-35 cells is strikingly similar to the requirement of an intact A-B-C arrangement for the human Hp element in human cells (42). Our results would also predict that certain constructs containing the human regulatory sequence are capable of being stimulated by IL-1, even though several studies with human hepatic cells have indicated that Hp expression is primarily controlled by IL-6 (41) and glucocorticoids (10, 13).

The distinct regulatory activity of the rat Hp sequence in rat and human cells (Fig. 2 and 4) is most likely tied to differences in the composition of *trans*-acting components. That the two cell types do not have equivalent sets of components is demonstrated by the substantial difference in their sensitivity to cycloheximide treatment (Fig. 1D) (5, 6). Obviously, the molecules constituting the IL-6 signal transduction pathway pre-exist in HepG2 cells and are not overly influenced by inhibiting protein synthesis. This suggests that a much greater abundance of the rate-limiting components is present in HepG2 cells than in H-35 cells. Preliminary characterization of the nuclear proteins from HepG2 and H-35 cells that interact with the rat Hp gene sequence has revealed substantial qualitative and quantitative differences between the cell types (data not presented). Furthermore, this finding is not limited to the Hp gene sequence; distinguishable cell-specific patterns have been noted for nuclear proteins interacting with the cytokine response elements of the genes for rat α_2 -macroglobulin (25) and rat α_1 -acid

glycoprotein (51). Therefore, by recognizing that cell-specific differences in regulation occur (Fig. 3) (37) and by anticipating the same occurrence for many transcription-controlling components, some caution should be exercised before accepting functional data derived from a single cell system for a given DNA sequence as conclusive. However, reproducing the same regulatory characteristics in separate systems (e.g., Fig. 2A and C and 5C) lends support for the involvement of a response element in the acute-phase reaction. A future goal will be to identify the specific components which are needed by the cytokines to generate a functional regulation through the promoter proximal rat Hp gene elements. By comparing our results with those obtained with other rat acute-phase protein genes (α_2 -macroglobulin [25, 27], angiotensinogen [48], and α_1 -acid glycoprotein [51]) and human genes (haptoglobin [42], hemopexin [46], serum amyloid A [16], C-reactive protein [35], and β -fibrinogen [26]), one might be able to decipher the molecular basis for coordinate control of these genetically unlinked and structurally unrelated genes.

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