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The Cytokine Response Element of the Rat α_1-Acid Glycoprotein Gene Is a Complex of Several Interacting Regulatory Sequences

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Expression of the rat α_1-acid glycoprotein gene is stimulated by interleukin-1 (IL-1) and interleukin-6 (IL-6) and is synergistically enhanced by the combination of the two. The distal regulatory element (DRE), a 142-base-pair (bp) sequence located 5 kilobase pairs upstream of the transcriptional start site, appears to be crucial for this cytokine response. The cytokine-specific regulatory sequences within the DRE have been identified by inserting individual DRE subregions, selected combinations of these, or a few linker mutated fragments into a plasmid containing an enhancerless simian virus 40 promoter linked to the chloramphenicol acetyltransferase gene. The regulatory activity was determined in transiently transfected human and rat hepatoma cells. The IL-1 response region was confined to the 5′-most 62 bp of the DRE, and its function seemed to depend on at least two separate components. The same region was also responsive to phorbol ester treatment. The IL-6 regulatory function was dependent on a 54-bp sequence located within the 3′ half of the DRE. When the IL-1 response region was recombined with the IL-6 regulatory region of the DRE or with IL-6 response elements of other plasma protein genes, a strong cooperative action by IL-1 and IL-6 was achieved. The functional DRE sequences were recognized by nuclear proteins extracted from rat liver and hepatoma cells. However, no cytokine-inducible binding activity was detectable, which suggests that transcriptional regulation through the DRE might be controlled by posttranslational modification of constitutively bound *trans*-acting factors.

The hepatic production of several plasma proteins, termed the acute-phase reactants, is increased after systemic tissue injury (34). In many vertebrate species, a common set of plasma proteins is similarly regulated (35). One of the major components is α_1-acid glycoprotein (AGP), which is thought to function as a nonspecific immunosuppressant (11) and may play a role in transporting cationic compounds (47). Recent development of hepatic tissue culture systems has helped to identify potential mediators of the hepatic acute-phase response (3). AGP gene expression is increased by interleukin-1 (IL-1), tumor necrosis factor α (TNF-α) interleukin-6 (IL-6), leukemia inhibitory factor (LIF), and glucocorticoids (9). Maximal stimulation in rat, mouse, and human hepatic cells requires a combination of at least IL-1, IL-6, and glucocorticoids (8, 9, 50). To elucidate the molecular mechanism of hormone action, the single-copy rat AGP gene has been cloned and characterized (54). Two separate regulatory regions have been recognized which are necessary and sufficient for reproducing the regulation pattern of the rat AGP gene in transiently transfected HepG2 cells (6, 49) and in transgenic mice (M. Dewey, C. Rheaume, F. G. Berger, and H. Baumann, J. Immunol. in press). The stimulation by glucocorticoids is mediated through a single glucocorticoid response element at positions −120 to −64 relative to the transcription start site (6). A 142-base-pair (bp) sequence, located between positions −5300 to −5150 and hence termed the distal regulatory element (DRE), is responsive to IL-1, TNF-α, IL-6, LIF, and phorbol ester (49). Molecular analyses of other acute-phase plasma protein genes have revealed *cis*-acting elements that respond to some of the same cytokines which also control the AGP gene. An IL-6 response element has been described for the genes encoding human and rat haptoglobin (42, 46), human hemopexin (48), human C-reactive protein (2), and rat α_2-macroglobulin (26). Moreover, an IL-6-inducible DNA-binding protein from human hepatoma cells that interacts with the IL-6 response elements from several human acute-phase protein genes has been found (40, 45, 48). Characterization of the genes for human serum amyloid A (18) and rat angiotensinogen (56) has implicated NF-κB or an NF-κB-like factor as a potential mediator for IL-1 regulation in HeLa or hepatic cells, respectively.

Since the DRE of the rat AGP gene is the target of several separate hormone signal pathways, a more precise definition of the functional sequences within that element is needed before one attempts a comparison with other gene elements. In this study, we have further dissected the DRE and document the presence of separable IL-1 and IL-6 response regions that show cytokine-specific regulatory activity in transiently transfected human and rat hepatoma cells. The data indicate that the interaction of several sequences in the DRE is required for achieving the optimal regulatory potential. Several functionally significant sequences in the DRE are recognized by nuclear proteins. Although there are subtle species-specific differences in the patterns of DRE-binding proteins, no cytokine-inducible binding component could be detected. If one or more of the DRE-bound proteins is involved in transcriptional regulation, the control of its function by the cytokine-dependent *trans*-acting factors might involve biochemical modification.

MATERIALS AND METHODS

Factors. Human recombinant IL-6 (10^6 B-cell stimulatory factor 2 units per mg) was prepared from the medium of monkey COS-1 cells that had been transfected with an expression plasmid, pCSF-309 (68); human recombinant

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IL-1α (10^8 U/mg) was obtained from Immunex Corp., Seattle, Wash. 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and 8-bromo-cyclic AMP (cAMP) were obtained from Sigma Chemical Co., St. Louis, Mo. TPA was dissolved in dimethyl sulfoxide at concentrations of 50 μg/ml.

Plasmid constructs. The simian virus 40 (SV40) promoter region from nucleotides 5171 to 101 (origin of replication plus three 21-bp repeats) was isolated from plasmid pAO (71) and inserted as a *Hin*dIII fragment into the *Hin*dIII site of pSVOCAT(Cla) (65) to generate pSV-CAT. The *Eco*RI-*Bal*I fragment containing the 142-bp rat AGP gene DRE (50) was inserted as a *Cla*I fragment into the *Cla*I site of pSV-CAT, yielding pDRE-SV-CAT. A separate set of plasmids containing DRE sequences was also prepared by using a pSV-CAT vector that carried an extra 6-bp linker sequence at the *Hin*dIII site (position 5020 in pSV2CAT [66]) and was used for the primer extension experiment (see Fig. 2D). Sequential 5′ and 3′ deletions of the DRE were made by digestion with BAL 31 exonuclease. Endpoints of the deletions and orientation of the inserts were identified by double-strand sequencing (32). Internal deletions and recombinations of DRE subfragments were derived from these 5′ and 3′ deletion constructs (see Fig. 3). p(10-37)-SV-CAT was constructed by inserting a synthetic oligonucleotide, representing the DRE sequence from positions 10 to 37, into the *Cla*I site of pSV-CAT. Plasmid p(10-37 mut)-SV-CAT contained the same oligonucleotide in which a thymidine at position 28 had been changed to a guanidine.

Plasmids pHp-Ad2-CAT and pFb-Ad2-CAT served as heterologous IL-6-responsive constructs used to test the synergistic action of the IL-1 response region of the DRE. pHp-Ad2-CAT obtained the IL-6 response element of the rat haptoglobin gene (−146 to −56) inserted in the *Sst*I-*Sma*I site 5′ to the major late promoter of adenovirus in pCT (42), and pβFb-Ad2-CAT contained the IL-6 response element of the rat β-fibrinogen gene (−192 to −43) in the *Xba*I site of pCT (H. Baumann et al., unpublished data).

Transient transfection. The standard procedure for testing the regulatory activity of gene elements was as follows. Twenty-four-hour cultures of HepG2 cells (31) in collagen-coated dishes were transfected with plasmid DNAs (15 to 20 μg per 10-cm dish) by calcium phosphate precipitation (25). After a 16-h recovery period, the cells were released by trypsin and equal amounts were plated into collagen-coated six-well cluster plates (4×10^5 cells per 10 cm^2). After 20 h, the medium was replaced by 1 ml of serum-free minimal essential medium (MEM) alone or containing 500 U of IL-1α per ml, 250 U of IL-6 per ml, or both. Twenty-four hours later, the culture medium was harvested and 50-μl samples were subjected to rocket immunoelectrophoresis (67). The relative change in production of the endogenous human AGP served as a measure for the fidelity of the cellular response to the cytokines. Chloramphenicol acetyltransferase (CAT) specific activity in the cell extract was determined (23). The effect of the cytokine treatment was expressed as fold stimulation and represented the ratio of the CAT activity in the treated cells to that of the untreated control cells.

Certain constructs were frequently included in transfection experiments during the past 2 years. The substantial variation in the collected data (see Fig. 3) can be explained in part by quantitative changes in HepG2 cell responsiveness to the cytokines as well as variation in the specific activities of IL-1 and IL-6 preparations used during the course of this study. Within the experimental series, however, relative changes in CAT activities were highly consistent (see Fig. 1, 3, 5, and 6). To measure basal-level expression of selected constructs, the cells were cotransfected with plasmid pIE-MUP (27, 37). The amount of mouse major urinary protein (MUP) produced and secreted into the medium was determined by rocket immunoelectrophoresis and was related to the CAT activity in the corresponding cell extracts (expressed as percent conversion of chloramphenicol to acetylated products per hour per nanogram of MUP [49]). H-35 cells (clone T-7-18 [55]) were transfected by the DEAE-dextran method (39). After recovery for 16 h, each cell culture was passaged into four 10-cm dishes. The hormone treatment and cell analysis were identical to those described for HepG2 cells.

RNA analysis. RNA from HepG2 cells was prepared by the guanidine thiocyanate procedure (14). Serial dilutions of RNA were dotted onto nitrocellulose and hybridized with cDNA inserts labeled by the random primer method (30).

The transcription start site of CAT constructs was determined by primer extension of a 5′-end-labeled synthetic 20-mer oligonucleotide (5′-TTTAGCTTCCTTAGCTCCTG-3′) which corresponds to the CAT gene region (positions 4987 to 5006 of pSV2CAT), as described previously (42).

Measurement of transcription rate. Nuclei were prepared from HepG2 cell monolayers in 10-cm plates by the procedure of Almendral et al. (1). The entire nuclear preparation was used for the run-on reaction according to the protocol of Lamers et al. (36). Equal amounts of ^{32}P-labeled RNA (5×10^7 cpm) were hybridized in 3 ml for 3 days to nitrocellulose strips carrying slot-blotted cDNAs (2 μg each) encoding human AGP (15), human haptoglobin (52), human α_1-antichymotrypsin (28), histone H4 (63), and the structural genes for human triosephosphate isomerase (41) and CAT. The magnitude of the transcription rate change was assessed by scintillation counting of the radioactivity associated with the individual probes and subtracting the background radioactivity of the filter. Since accurate initiation or termination of CAT plasmid transcription did not seem always to occur, a low level of transcribed vector sequences appeared in the nuclear RNA preparations and gave rise to a hybridization signal with pBR322 DNA.

Nuclear extracts. Buffalo rats (3 months old, weighing 300 g) were subcutaneously injected with 250 μl of turpentine to induce an acute-phase reaction. At various times, the liver was removed and nuclear extracts were prepared as described by Gorski et al. (24). Nuclear extracts from HepG2 cells were prepared by the procedure of Shapiro et al. (60).

DNase I protection assays and gel retardation analysis. DNA-binding reactions and DNase I digestions were carried out essentially as described previously (16, 24). The specific DRE probes for gel retardation analysis were prepared from plasmid pDRE-SV-CAT, pAB-SV-CAT, pC-SV-CAT, or pD-SV-CAT. Binding reactions with nuclear proteins were carried out as for the DNase I protection assays. One half of the reaction mixture was loaded directly onto 5% polyacrylamide gels and electrophoresed at 4°C in 25 mM Tris (pH 8.3)–25 mM boric acid–0.5 mM EDTA. For competition experiments, the unlabeled DNA fragments (DRE, AB, C, and D) were added to the reaction mixture before addition of the nuclear extract.

RESULTS

Activity of the DRE. Previous characterization of the DRE function was primarily performed in CAT expression vectors containing the AGP gene promoter whose activity was controlled additionally by a promoter-proximal glucocorticoid response element (6). To achieve an unambiguous

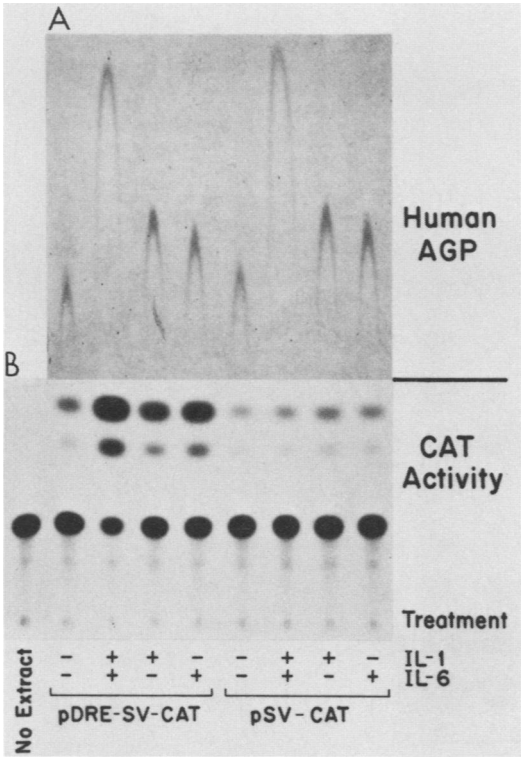

FIG. 1. Cytokine-specific regulation of DRE-containing plasmids. HepG2 cells were transfected with 15 μg of pDRE-SV-CAT or pSV-CAT. Cells were trypsinized at 16 h after transfection, divided into four cultures, and treated with serum-free medium alone or containing 500 U of IL-1 per ml 250 U of IL-6 per ml, or both. After 24 h, the CAT activity in the cell extract (B) and the amount of human AGP in the medium (A) were determined.

assessment of DRE activity, the 142-bp DRE was inserted into a CAT vector upstream of a minimal SV40 promoter. The plasmid DNA was then tested for cytokine-specific expression by transient transfection into HepG2 cells (Fig. 1). Either IL-1 or IL-6 stimulated CAT activity between 3- to 5-fold and, when combined, mediated a 10- to 15-fold increase (Table 1; see also Fig. 3, construct 1). The cytokine regulation of the transfected plasmid was qualitatively similar to that of the endogenous human gene, as judged from the change in the amounts of secreted AGP (Fig. 1). The presence of the DRE sequence enhanced the basal-level expression of the vector by threefold (Table 1; see also Fig. 3, constructs 1 and 24), suggesting that the DRE not only acts as a cytokine response element but also contains an enhancer activity. The latter feature is more pronounced with plasmids containing duplicate copies of the DRE. The regulatory capacity was maintained after inversion of the DRE sequences. The overall magnitude of cytokine stimulation was not, however, improved either by duplication or by reverse orientation (Table 1).

TABLE 1. Orientation- and location-independent action of the DRE[a]

Construct	Specific CAT activity (% converted/h per μg of protein)		Fold stimulation
	Untreated	IL-1 + IL-6	
pDRE-SV-CAT	0.6	7.0	11
p2xDRE-SV-CAT	1.5	10.3	7
p2xR-DRE-SV-CAT	3.2	8.6	3
pDRE(BamH1)-SV-CAT	0.4	1.7	4
p2xDRE(BamH1)-SV-CAT	0.9	10.1	11
p2xR-DRE(BamH1)-SV-CAT	1.0	7.9	8
pSV-CAT	0.2	0.3	1

[a] The indicated plasmids were transfected in parellel into HepG2 cells. The cells were divided into two dishes, which were then treated for 24 h with either serum-free medium alone or medium containing 500 U of IL-1 and 250 U of IL-6 per ml.

To assess whether the DRE functioned over distance, the element was also inserted into the *Bam*HI site at the 3′ end of the CAT gene, 1.6 kilobase pairs downstream (or 2.9 kilobase pairs upstream) of the SV40 promoter. The ability of the DRE to confer responsiveness to IL-1 and IL-6 was preserved and was again independent of orientation (Table 1). We concluded from these results that the 142-bp DRE sequence is a cytokine response element that also fulfills the basic properties of an enhancer.

DRE controls transcriptional activity of the CAT vector. The transcriptional regulation of pDRE-SV-CAT in transiently transfected HepG2 cells is shown by a direct comparison of the cytokine-induced changes in run-on transcription, mRNA levels, and CAT activity (Fig. 2A and B). In this experiment, the relative amounts of run-on transcripts were 3.3-fold by 4 h and 4.7-fold by 24 h of treatment (Fig. 2A). These values were in close agreement with the relative change in the CAT mRNA and CAT activity (Fig. 2B), corroborating that the cytokines acted primarily at the level of transcription. The temporal change in CAT gene expression was comparable to those of the endogenous genes such as the AGP or haptoglobin gene (Fig. 2C). We determined by primer extension analysis that the correct transcription start sites of the transfected plasmids were used in both control and cytokine-treated cells (Fig. 2D). Anticipating low concentrations of CAT mRNA in untreated HepG2 cells, we used CAT expression vectors containing two copies of the DRE for transfection to ensure an elevated basal level of the transcripts. The two major primer extension products were obtained with polyadenylated RNA preparations from untreated and treated cells which migrated with sizes of 98 and 102 nucleotides. These sizes were predicted on the basis of the known two transcription start sites within the SV40 promoter sequence. Since the experiment in Fig. 2D was carried out with nonnormalized preparations of polyadenylated mRNA, the autoradiogram did not allow quantitation of the cytokine-induced changes in CAT mRNA concentration. The accuracy of our determination was verified by demonstrating that the extension products of the pSV2CAT-derived transcripts were 6 nucleotides shorter (92 and 96 nucleotides), as predicted. The transcription start sites were the same regardless of cell treatment or orientation of the DRE insert.

Identification of the IL-1- and IL-6-specific regulatory sequences within the DRE. Further delineation of the specific regulatory regions within the DRE was approached by creating a series of progressive 5′ and 3′ deletions and by testing the functionality of the remaining DRE sequences in transiently transfected HepG2 cells (Fig. 3). Deletion of the first 25 bp at the 3′ end (construct 2) led to a twofold reduction of the IL-6 response. Removal of an additional 55 bp (from construct 3 to 5) resulted in a gradual loss of IL-6 regulation. The IL-1 response, although slightly diminished, was maintained in those constructs unless the deleted region extended from 29 to 142 (construct 7). Functional tests of the

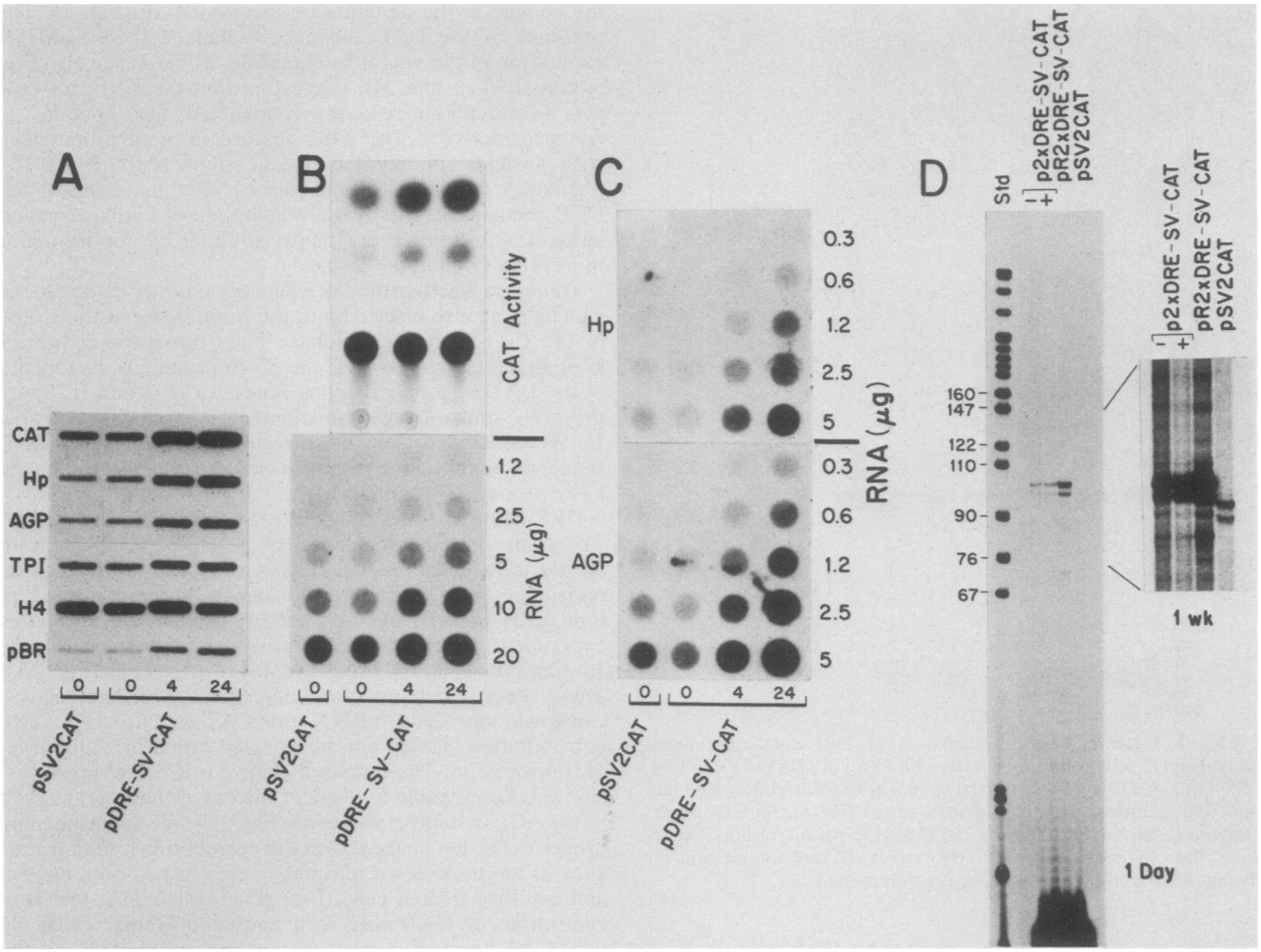

FIG. 2. Cytokine-induced changes in gene expression in transiently transfected HepG2 cells. pSV2CAT and pDRE-SV-CAT plasmid DNAs were transfected into HepG2 cells in two and six 10-cm dishes, respectively. After 36 h, the media were replaced by serum-free minimal essential medium. IL-1 and IL-6 were added immediately or 20 h later to a duplicate set of plates. After 24 h, a quarter of the monolayer with cells transfected with pDRE-SV-CAT was removed from one dish and used to measure CAT activity. The remaining cells from the duplicate plates with 0-, 4-, and 24-h cytokine treatments were combined, and the nuclei were prepared. (A) Run-on transcripts (5×10^7 cpm each) were detected by hybridization to immobilized CAT gene and to cDNAs for human haptoglobin (Hp), human AGP, human triose phosphate isomerase (TPI), histone H4 (H4), and pBR322. An autoradiogram after 6 h of exposure is shown. (B and C) Total RNA was isolated from the cell lysates after removal of the nuclei; serial dilutions of the RNA were dot blotted onto nitrocellulose and probed with ^{32}P-labeled CAT gene (B, bottom) or cDNAs for human AGP and haptoglobin (C). Autoradiograms were exposed for 24 h. The relative change in CAT activity is compared with the change in CAT mRNA in panel B. (D) Primer extension analysis of poly(A)$^+$ RNA from HepG2 cells transfected with pDRE-SV-CAT and treated for 24 h with serum-free medium alone (−) or containing IL-1 and IL-6 (+, and where not indicated). Total cellular RNA from cells transfected with pSV2CAT served as a control template. The same gel was exposed for 1 day and 1 week. *Hpa*II-digested pBR322 served as molecular size standards (Std).

deleted regions (constructs 9 to 12) failed to reveal significantly regulatory function except for the minor IL-6 response noted for constructs 9 to 11. The overall regulation appeared to depend critically on the first 28 bp at the 5′ end, since deletion of this sequence virtually extinguished cytokine regulation (construct 9). However, the 28-bp fragment on its own was devoid of a regulatory element (construct 7). From these deletion analyses, we concluded that an IL-6 regulatory sequence resided in the 3′ half and an IL-1 regulatory sequence in the 5′ half of the DRE.

To more clearly define these *cis*-acting sequences, the DRE was subdivided into the four segments, A to D. Each segment alone, either as a single copy or as an oligomer, or in combinations was functionally tested (constructs 5 to 9 and constructs 12 to 20). IL-1-specific regulation was recovered in fragment AB, whose regulatory activity was magnified upon oligomerization (construct 6). Since neither fragment A nor fragment B alone showed any regulatory activity, the function of the joint AB fragment could be explained by the action either of two separate but strongly synergizing elements or of a single regulatory sequence residing at the AB junction. The latter possibility was supported by the finding that only a colinear arrangement of the AB segments was functional; an insertion of 8 bp at the AB junction rendered the AB sequence inactive and, when present in the DRE, reduced the responsiveness of that element to the levels observed for construct 9, 19, or 20. However, the test of a synthetic 28-bp oligonucleotide, representing the AB junction sequence (as well as the major protein-binding site in the AB segment; see Fig. 6) yielded

Construct No.	DRE Sequence		Fold Stimulation of CAT Activity: IL-1 + IL-6	IL-1	IL-6	Basal Level Activity
1	1 - 142	EcoR1 Hinf1 Bal1	13.0 ± 3.5 (11)	3.3 ± 1.3 (9)	4.5 ± 1.4 (9)	0.12
2	1 - 117		6.3 ± 2.7 (5)	2.5 ± 0.9 (3)	2.8 ± 1.0 (3)	
3	1 - 88		5.1 ± 0.8 (3)	3.2	2.0	
4	1 - 82		4.4 ± 1.3 (5)	2.2 ± 0.3 (3)	1.5 ± 0.1 (3)	
5	1 - 62	A B	3.0 ± 1.7 (3)	2.7 ± 2.2 (3)	1.1 ± 0.3 (3)	0.10
6	4 x (1 - 62)	4 x A B	52 ± 33 (3)	28 ± 16 (3)	3.8 ± 2.0 (3)	
7	1 - 28	A	0.4 ± 0.1 (3)	0.8 ± 0.1 (3)	0.7 ± 0.3 (3)	
8	3 x (1 - 28)	3 x A	1.3 ± 0.6 (3)	1.1 ± 0.7 (3)	1.0 ± 0.5 (3)	
9	29 - 142		1.9 ± 0.9 (4)	1.1 ± 0.8 (3)	1.6 ± 0.6 (3)	
10	60 - 142		2.0 ± 0.3 (3)	1.2	1.6	
11	78 - 142		1.4 ± 0.1 (3)	1.1 ± 0.1 (2)	1.4 ± 0.1 (2)	
12	118 - 142	D	0.6 ± 0.2 (3)	1.0 ± 0.7 (3)	0.8 ± 0.3 (3)	
13	3 x (118 - 142)	3 x D	0.6	0.5	1.0	
14	29 - 62	B	0.8 ± 0.4 (3)	1.2 ± 0.6 (3)	1.0 ± 0.4 (3)	
15	4 x (29 - 62)	4 x B	0.7	1.4	0.7	
16	63 - 117	C	2.5 ± 0.4 (4)	1.3 ± 0.4 (4)	2.8 ± 0.9 (4)	0.09
17	2 x (63 - 117)	2 x C	6.6 ± 1.1 (3)	1.7 ± 0.5 (3)	6.1 ± 0.5 (3)	
18	29 - 117	B C	1.4 ± 0.6 (2)	1.4 ± 0.8 (2)	0.9 ± 0.1 (2)	
19	1 - 28 / 63 - 117	A C	2.5 ± 1.3 (2)	1.4 ± 0.1 (2)	2.9 ± 1.2 (2)	
20	1 - 28 / 63 - 142	A C D	2.5 ± 2.5 (3)	2.5 ± 1.8 (3)	2.8 ± 1.8 (2)	
21	10 - 37		1.5	1.0	1.2	
22	3 x (10 - 37)	3 x	2.3	1.4	1.2	
23	3 x (10 - 37 mut)	3 x mut	0.6	1.2	0.8	
24	vector		1.1 ± 0.4 (8)	1.3 ± 0.5 (9)	1.1 ± 0.4 (9)	0.04

FIG. 3. Localization of functional regions within the DRE. All indicated DRE fragments were cloned into the *ClaI* site of the pSV-CAT vector as described in Materials and Methods. The first nucleotide of the DRE was designated 1, and the last was designated 142 (see Fig. 10 for sequence). The ability to be regulated by IL-1 and IL-6 was assessed in transiently transfected HepG2 cells. Specific CAT activity (percent conversion per hour per microgram of cell protein) was determined for each culture and then expressed as a ratio to the untreated control culture for each experimental series. The means and standard deviations are shown when three or more independent experiments had been done, and the means and ranges are shown when only two independent experiments had been done. Basal-level activity was determined in separate experiments by cotransfection with pIE-MUP (see Materials and Methods). Values represent percent conversion per hour per nanogram of MUP for untreated cells.

only a minimal IL-1 response (constructs 21 and 22). This activity, if significant, was completely eliminated by a T-to-G substitution at position 28 (construct 23). On the basis of this finding, we concluded that the IL-1 regulatory function was not manifested by a single oligonucleotide sequence but depended on interacting elements spanning the first 62 bp of the DRE.

An IL-6-specific regulation was observed with fragment C, which as a single copy could account for about one-half of the IL-6 response of the entire DRE (construct 16). The activity was not improved by addition of either adjacent fragment B or D (constructs 18 and 10). The low activity of the latter construct was surprising considering that deletion of fragment D from the DRE reduced preferentially the IL-6 regulation. As found for other regulatory sequences, dimerization of fragment C increased the magnitude of the IL-6 response (construct 17).

Taken together, the functional analysis of DRE subregions presented in Fig. 3 indicated that separable IL-1- and IL-6-specific regulatory sequences are present, but that maximal regulation requires an interaction of the two sequences with additional, contributing activator sequences.

Regulation of DRE regions by TPA. Recent studies have suggested that intracellular IL-1 signal transduction involves cAMP in YT, 3T3, and 70Z/3 cells (62) or an activated phospholipid pathway in Jurkat cells (57). Activated protein kinase C has been implicated in mimicking the action of IL-6 in hepatic cells (19) and acute-phase regulation of serum amyloid gene elements in transfected HeLa cells (18). The interleukin-specific gene constructs in Fig. 3 are well suited to probe the effect of the proposed messenger systems in mediating the cytokine regulation in HepG2 cells. Previously, we have shown that in HepG2 cells the DRE linked to the AGP gene promoter responds to TPA (4) but not to 8-bromo-cAMP (Baumann, unpublished data). Extending those tests to CAT plasmids containing four copies of AB (construct 6), four copies of B (construct 15), two copies of C (construct 17), three copies of D (construct 13), and three copies of segment 10-37 (construct 22), only the first construct responded to TPA significantly above the vector alone

TABLE 2. Effect of different mediators on the expression of IL-1 response element-containing plasmid[a]

Treatment	Fold stimulation of CAT activity
IL-1	13.2
IL-6	2.5
8-bromo-cAMP	0.9
TPA	7.1
IL-1 + TPA	25.0
IL-6 + TPA	34.3
IL-1 + IL-6	31.0

[a] HepG2 cells were transfected with p4xAB-SV-CAT plasmid DNA. After 16 h of recovery, the cells were divided into eight cultures. Twenty-four hours later, these cultures were treated for 8 h with serum-free medium alone or containing 500 U of IL-1 per ml, 250 U of IL-6 per ml, 0.1 mM 8-bromo-cAMP, or 0.25 μM TPA. The CAT activity in the cell extract was determined and expressed as a fraction relative to the untreated control culture. Mean values of two separate experiments are shown. The vector pSV-CAT similarly transfected and tested for TPA response yielded a relative value of 1.75.

(Table 2; data for the other plasmids are not shown). Although both IL-1 and TPA stimulated expression of the AB-containing plasmid severalfold, their action appeared to be separate because a combination of the two yielded an additive response. More surprising, however, was the strong synergistic action between IL-6 and TPA, which was comparable to that of IL-6 and IL-1. It appears that the intracellular IL-6 signal can be effectively directed to the AB element but only when the cells have been costimulated with either IL-1 or TPA.

Interaction of the IL-1-responsive AB region with heterologous IL-6 response elements. Although a single-copy AB region showed relatively low regulating activity, its action was magnified when the region was linked to the IL-6-responsive C region and then tested with the combination of the two cytokines (compare constructs 5 and 2 in Fig. 3). This cooperativity was not restricted to the DRE regions but was also apparent when the AB region was combined with the IL-6 responsive elements of other genes (Fig. 4). When the AB region was inserted 5′ to the IL-6 regulatory element of the rat haptoglobin gene, which on its own is not responsive to IL-1 and whose IL-6 stimulation is even reduced twofold by IL-1 (Fig. 4; 42), a 10-fold-stimulated expression of the AB-haptoglobin constructs was achieved by IL-1. Moreover, the IL-6 response was enhanced twofold, yielding a 29-fold stimulation. The combination of IL-1 and IL-6 produced a 100-fold increase in CAT activity. A qualitatively similar but quantitatively less pronounced modification of the cytokine response pattern was observed with a combination of the AB region with the IL-6 response element of the rat β-fibrinogen gene (Fig. 4). This observation suggests that the cytokine-specific regulation of acute-phase plasma protein genes might be in part dependent on the modular arrangements of IL-1- and IL-6-specific elements.

Regulation of the DRE regions in rat cells. Since the cytokine-specific responses of the AB and C regions have been defined in HepG2 cells, the question arose as to whether these DRE segments also functioned similarly in rat hepatic cells. We chose H-35 cells as a test system because these cells have been found to respond to cytokines by changing the expression of specific sets of acute-phase plasma proteins (Fig. 5A; 7). We transfected the SV-CAT plasmids containing either four copies of AB, two copies of C, or one copy of the DRE into these cells and determined the cytokine-dependent change in CAT expression (Fig. 5B). The AB-fragment-containing plasmid was fivefold stimulated by either IL-1 or IL-6, indicating that the activity of the AB

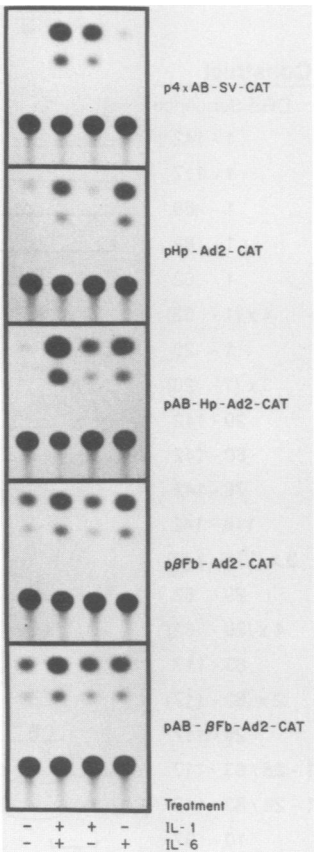

FIG. 4. Function of the IL-1 response region of the DRE. The indicated plasmids were transfected in parallel into HepG2 cells. The subcultures were treated with cytokines for 24 h. Equivalent amounts of cell extracts were used for measuring CAT activity.

element was less IL-1 responsive in H-35 cells than in HepG2 cells (Fig. 3, construct 6). The combination of IL-1 and IL-6 yielded an overall 15-fold stimulation.

Cells transfected with the C-fragment-containing plasmid showed CAT activity increased fourfold by IL-6. IL-1 alone did not notably effect CAT expression, nor did it modify the IL-6 response. The regulation pattern for the DRE-containing plasmid was qualitatively similar to that in HepG2 cells except that the magnitude of cytokine stimulation was much reduced. Most prominent was the relatively high basal-level expression of pDRE-SV-CAT in H-35 cells. This comparison of DRE-CAT constructs in rat and human hepatoma cells indicated that the regulatory sequences of the DRE functioned appropriately in both cell systems but that there were substantial differences in *trans*-acting elements, which contributed to altered basal-level expression, magnitudes of stimulation, and relative responsiveness to IL-1 and IL-6.

Interaction of nuclear proteins with the DRE. Having identified the functional regions of the DRE, we assessed whether these regions could serve as binding sites for specific nuclear proteins. To do so, proteins were extracted from liver nuclei of a control and a 13-h acute-phase rat. The sites of protein binding to the DRE sequence was visualized by DNase I footprinting, using a duplicated DRE as a probe (Fig. 6A). The repeated footprint pattern was taken as evidence for the specificity of protein binding. Four major nuclease-protected regions were observed, which coincided

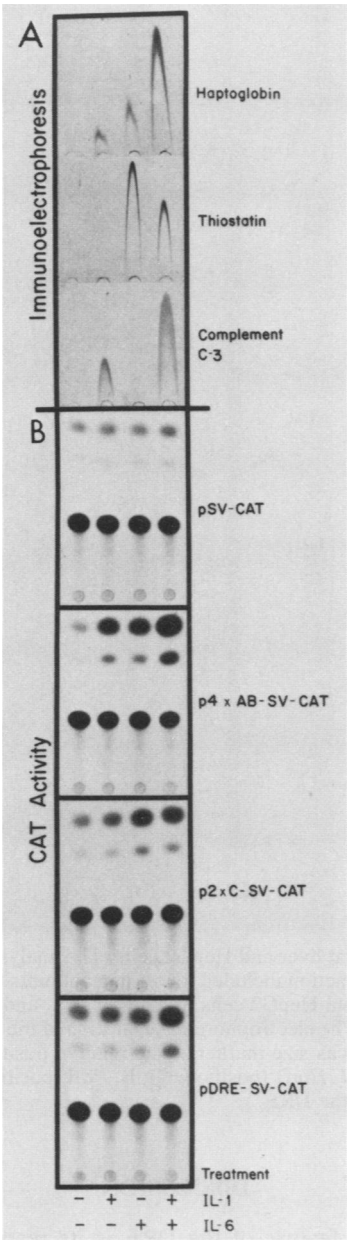

FIG. 5. Activity of the DRE region in rat hepatoma cells. H-35 cells were transfected with indicated plasmids and, after subculturing, treated with cytokines. Equivalent amounts of cell extracts were assayed for CAT activity. The amount of haptoglobin, thiostatin, and complement C3 secreted during the 24-h cytokine treatment period was determined by rocket immunoelectrophoresis.

with those detected with a single-copy DRE (Fig. 6B). These regions correspond to the DRE sequences 7 to 38, 48 to 56, 76 to 97, and 128 to 142. The first two binding sites were located in the AB region, the third was in the C region, and the fourth was in the D region. The nuclear proteins from control and acute-phase liver gave virtually identical nuclease protection patterns. Moreover, no obvious quantitative differences in binding activities appeared to exist between the two liver extracts (Fig. 6A).

The same DNase I digestion protocol was applied to the analysis of nuclear proteins from HepG2 cells extracted at various times after stimulation with IL-1 and IL-6 (Fig. 6C). Most notable was the broad protected region within the AB region at positions 10 to 38. Under the conditions used, nuclease protection at the other regions, around 50, 80, and 130, were less apparent. Although there were quantitative differences in binding activities between rat and human extracts, qualitative similarities in DRE sequence recognition also seemed to exist.

To evaluate more precisely the quantitative and qualitative changes in DRE-binding proteins, the interaction of nuclear proteins with DRE subfragments was determined by gel mobility shift. Proteins were extracted from liver nuclei at various stages of the acute-phase response. The time points coincided with increased transcriptional activity of several acute-phase plasma protein genes, including the AGP gene (22, 33). Proteins from control liver produced two major complexes with the entire DRE sequence (Fig. 7A). Extracts from 4- to 12-h acute-phase liver yielded an additional, faster-migrating complex (arrowhead in Fig. 7). This particular binding activity was substantially reduced at 16 h and absent at 24 h of the acute phase. The relative concentration of the binding activities at 0 and 4 h was quantitated by testing increasing amounts of extracts (Fig. 7B). None of the untreated liver extracts showed the additional faster-migrating complex at any of the concentrations tested. Two separate extracts of control liver nuclei and four of acute-phase nuclei were analyzed, and the results were identical to those obtained for the representative series in Fig. 7A and B.

To achieve a protein binding to the DRE subfragments, higher concentrations of nuclear protein were required. Both fragments AB and C produced several separable complexes whose electrophoresis patterns were essentially constant during the course of the acute phase. The band differences occasionally observed with fragment C, however, could not consistently be reproduced and failed to show any correlation with a specific stage of the acute phase.

The contribution of each subfragment to the protein-binding pattern of the entire DRE was identified by competition experiments (Fig. 7C). Formation of the faster-migrating protein complexes with the DRE was completely prevented by excess amounts of unlabeled DRE or fragment AB. However, the prominent, slower-migrating complex was only partially affected. Excess amounts of fragment C or D did not influence the overall binding, suggesting a minor contribution from these sequences. Similar results were obtained for the protein binding to the AB fragment; both DRE and AB were equally effective in competing for binding (Fig. 7C). From these binding studies, we concluded that rat liver nuclei contain proteins that bind to specific sequences of the DRE, with a major binding site in fragment AB. An acute-phase-dependent, minor change in protein-binding patterns was detectable only when probed with intact DRE.

Since the liver is controlled in vivo by a large variety of effectors during the course of the acute-phase reaction, we asked whether in the well-defined tissue culture system, the cytokine treatment was also correlated with specific changes in DRE-binding activities. Therefore, nuclear proteins were prepared from HepG2 cells after IL-1 and IL-6 treatment and tested for DRE binding by gel mobility shift assay (Fig. 8A). Although the total DRE and each separate fragment produced a multicomponent pattern, the compositions of these were not detectably influenced by cytokine treatment. As found with the rat liver proteins, the faster-migrating complexes of HepG2 cell proteins obtained by using the DRE or AB fragment could be competed for by excess unlabeled DRE or AB fragment (Fig. 8B). It appears that protein interactions with the AB region contribute most to the

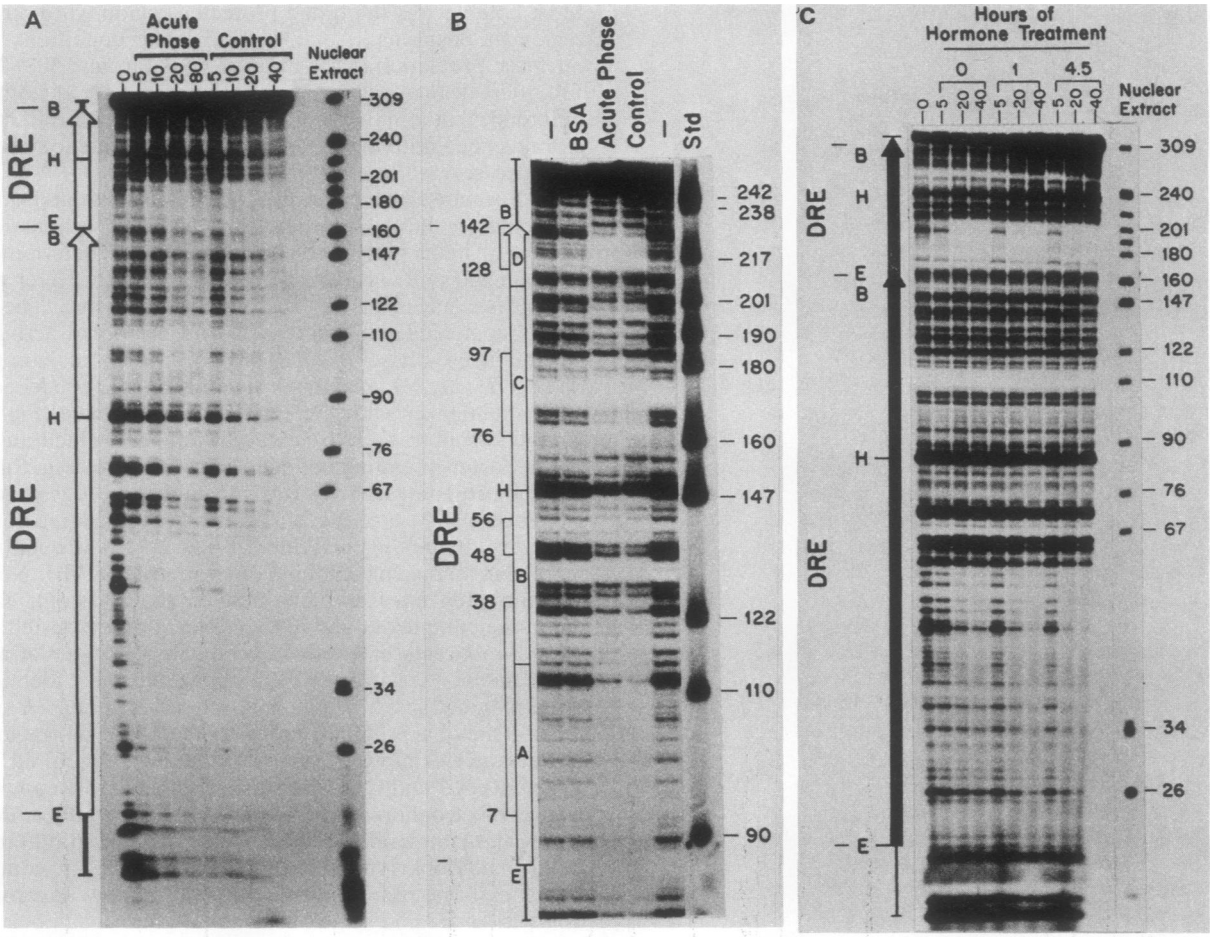

FIG. 6. DNase I footprinting analysis of DRE interacting with nuclear proteins from rat liver and HepG2 cells. The analyses were carried out on the coding strand of the double DRE (A and C) or single DRE (B). The binding reaction included increasing amounts (in micrograms) of nuclear protein extracted from untreated and 13-h acute-phase liver (A and B) and from HepG2 cells treated for 0, 1, and 4.5 h with IL-1 and IL-6 (C). In panel B, 25 μg of bovine serum albumin or nuclear extracts was applied. The electrophoretic separation of the DNase I digests on 8% denaturing polyacrylamide gels (44) included *Hpa*II-digested pBR322 DNA (Std) as size markers. The relative position of the DRE sequence is shown at the left. Relevant restriction enzyme sites: *E*, *Eco*RI (position 1); *H*, *Hin*fI (position 62); B, *Bal*I (position 142). DNase I-protected regions are indicated by brackets along with flanking nucleotide number of the DRE.

complex formation observed with entire DRE sequence. The preferential binding to that region was supported by the finding with the DNase I protection assay (Fig. 6C).

Comparison of DRE-binding proteins from liver and hepatoma cells. The electrophoretic pattern of the DRE-protein complexes appeared to be quite different when we compared the results of liver and HepG2 cell extracts (Fig. 7A and 8A). However, side-by-side separation of complexes formed with nuclear extracts from liver, H-35, and HepG2 cells revealed comigrating bands (Fig. 9). Most obvious were quantitative differences: liver and H-35 cell proteins generated an abundant slow-migrating complex that was barely detectable with HepG2 cell extract. H-35 and HepG2 cell extracts yielded a much higher amount of a fast-migrating band that coincided in position with the band inducible by the acute phase in liver. However, in neither H-35 nor HepG2 cells was the relative abundance of that complex-forming protein affected by cytokine treatment (Fig. 8C; data not shown for H-35 cells). It is evident from this comparison that considerable variation exists in DRE-binding proteins from different cell types. A major task will be to identify which of these interactions is causal to the transcriptional regulation.

DISCUSSION

The unique feature of the DRE is its responsiveness to several distinct cytokines. It thereby has the potential to contribute significantly to the transcriptional regulation of the AGP gene during an acute-phase reaction (33; Dewey et al., in press). Considering that several other rat acute-phase plasma protein genes are similarly regulated by the same set of cytokines as the AGP gene (7), a common molecular basis for the coordinate control must exist. Therefore, a comparison of the principal *cis*-acting regulatory elements of the various acute-phase protein genes might indicate whether the shared usage of similar *trans*-acting elements is probable. Such a cross-comparison has been facilitated by the recent wave of published reports on cytokine response elements in rodent and human genes.

This study demonstrates that the 142-bp DRE (Fig. 10A) requires several segments for optimal cytokine regulation. The regions conferring IL-1- and IL-6-specific responses (sequences from 1 to 62 and 63 to 117) are separable, and their cytokine specificity action is amplified by oligomerization. Similar observations have been made by others during

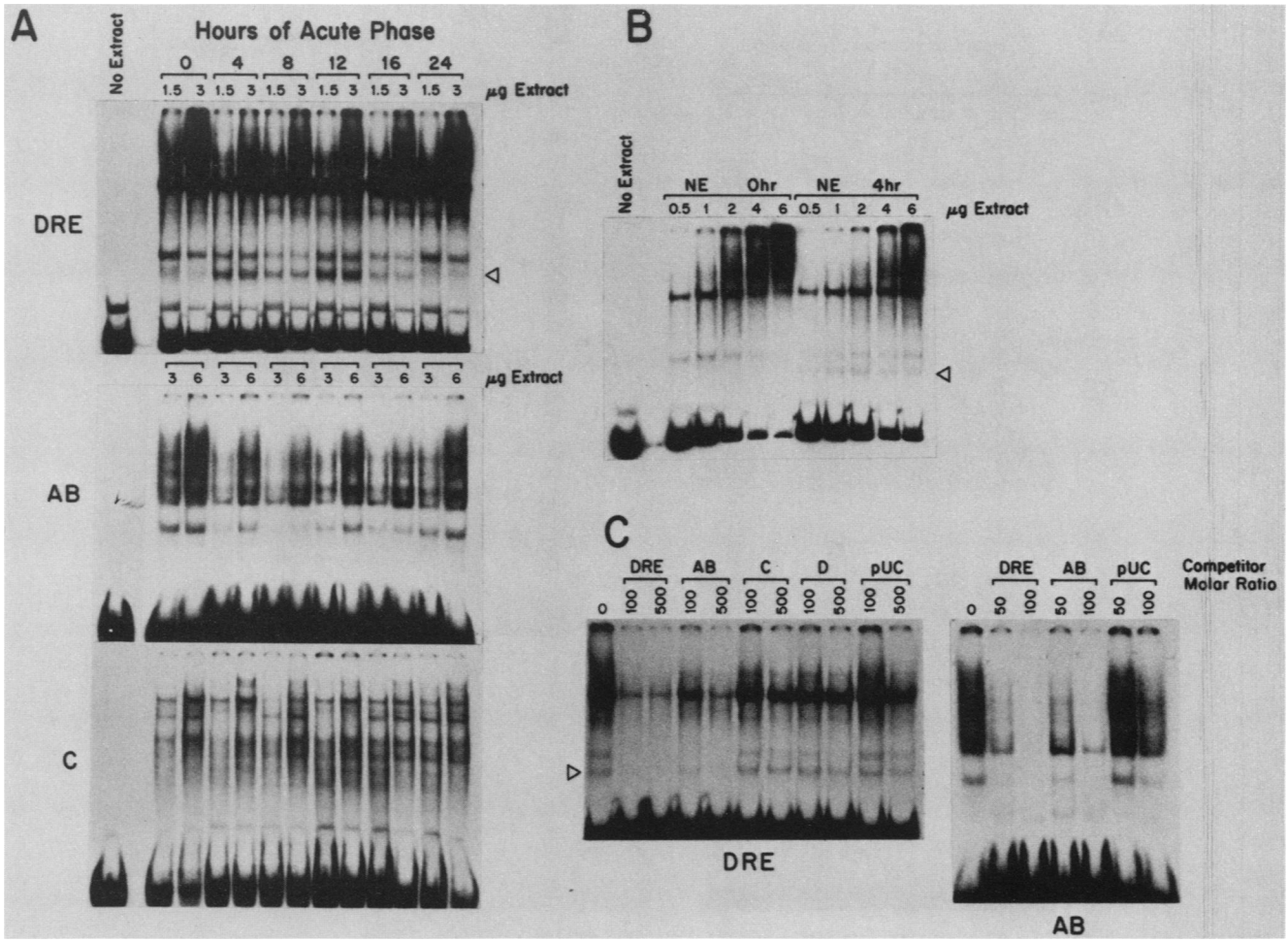

FIG. 7. Gel retardation analysis with rat liver nuclear extracts. (A) Effect of acute phase on binding activity on the total DRE, fragment AB, and fragment C. The amount of protein included in the binding reaction is shown at the top of each lane. (B) Titration of binding activity in the nuclear extract from untreated liver (NE 0 hr) or 4-h acute-phase liver (NE 4hr). Total DRE was used as a probe. (C) Binding of nuclear extract from 4-h acute-phase liver to the DRE and fragment AB, probed with indicated molar excess of unlabeled DRE, fragments AB, C, and D, and pUC13. The arrowhead indicates an additional complex that appeared when acute-phase liver extract was used.

their attempts to pinpoint the minimal cytokine response element for various acute-phase protein genes (40, 45, 48). This apparent requirement for multiple components in order to obtain functional cytokine regulatory elements is in agreement with the findings on other types of hormone response or enhancer elements, indicating that the interaction of several transcription-controlling elements is crucial for generating proper functionality (17).

The functional regions within the DRE coincide with nuclear protein-binding sites (Fig. 10A), a property which enhances the validity of comparison to equivalent elements of other genes. Although the IL-6 response of the entire DRE is only a fewfold (Fig. 3), the C fragment yielded an activity similar in magnitude to those of subcloned segments of the IL-6 response elements of the rat (42) and human (45) haptoglobin genes. The sequence motif within the C fragment at positions 82 to 90, which is also recognized by nuclear protein, is most similar to the type B sequence found in other acute-phase protein genes (Fig. 10B). The functional significance of that sequence has been demonstrated by site-specific mutation analysis in the case of rat α_2-macroglobulin (26) and human haptoglobin (45). A second, type B-like sequence is located in inverted orientation at positions 3 to 11 in fragment A but seems not to act as a regulatory element when tested as a single copy or as a multimer, nor did it enhance the IL-6 responsiveness of fragment C (Fig. 3).

The dependence of fragment C on other DRE regions to achieve maximal activity is similar to the findings with rat and human haptoglobin genes (42, 45). In those cases, the functional IL-6 response element requires a second sequence of the type A motif in addition to the type B motif. This type A sequence was also found associated with regulatory regions of the other IL-6-sensitive genes (Fig. 10B). Indeed, the DRE contains two type A-like sequences, but those reside in the A and B fragments, respectively. Either or both might contribute to the low-level IL-6 response of the AB fragment or of the entire DRE (Fig. 3), although these sequences fail to function as a response element on their own. Since the type A sequence of the human haptoglobin gene has been shown to be a prominent protein-binding site, it is likely that the equivalent DRE sequence also binds nuclear factors, thereby explaining the broad protein-binding region in the AB segment (Fig. 6).

Deletion analyses have indicated that the 3'-most sequence of the DRE, or fragment D, is important for full IL-6

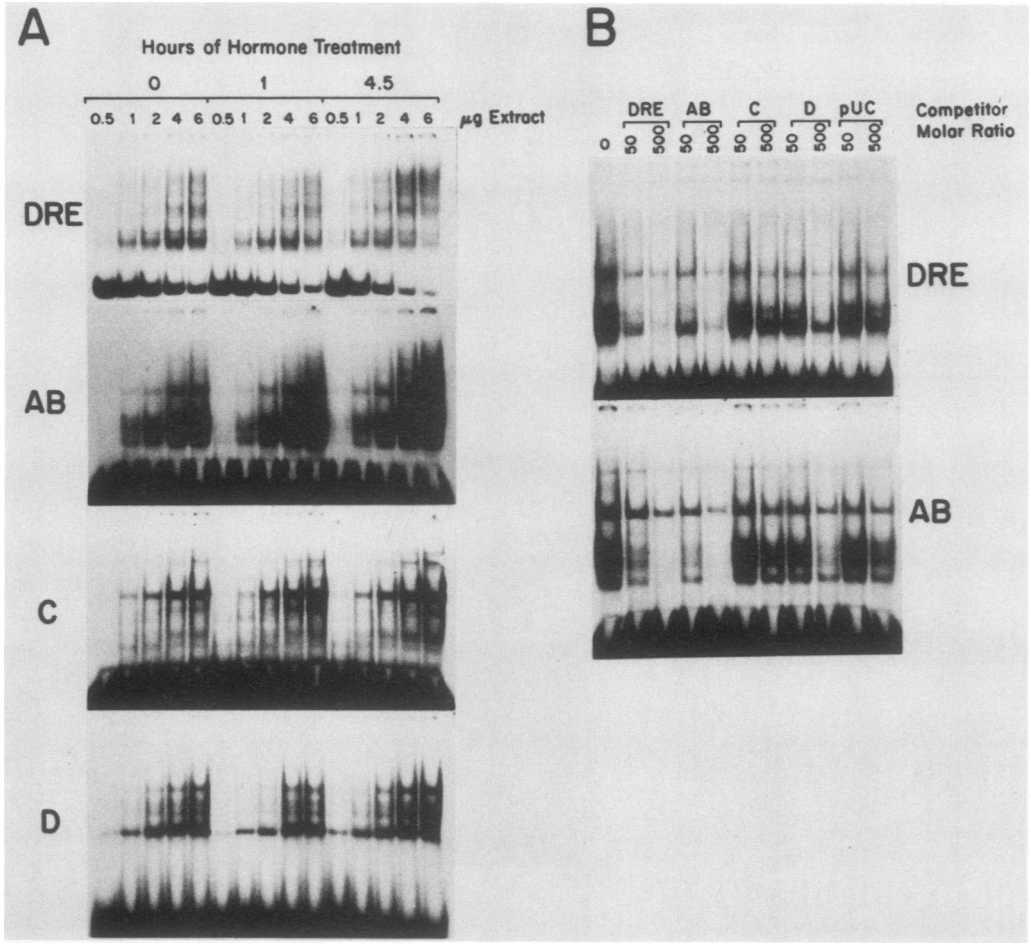

FIG. 8. Gel retardation analysis with HepG2 cell nuclear extracts. (A) The nuclear extracts were prepared from untreated cells or from cells treated with IL-1 and IL-6 for 1 or 4.5 h. ^{32}P-labeled DRE and fragments AB, C, and D were incubated with the indicated amounts of cell nuclear extracts. (B) Binding of nuclear proteins from 4.5-h treated HepG2 cells to labeled DRE or fragment AB was carried out in the presence of the indicated molar excess of unlabeled competitors.

regulation of the DRE (Fig. 3). We can only speculate that this sequence, which is also a prominent protein-binding region in the context of the DRE (Fig. 6), fulfills a contributing role in establishing an active cytokine element as ascribed to the type B motif sequence of the human haptoglobin gene (45). Surprisingly, this sequence, termed type C, is highly similar to one present in the IL-6 response element of the rat haptoglobin gene (Fig. 10B; 42). Considering that a combination of fragment D with C does not improve the IL-6 response of the latter, a more complex interaction of subregions must be operative in the DRE rather than just an additive effect.

The AB fragment acts as an IL-1 response element and is functionally comparable to the acute-phase regulatory elements which are related, if not identical, to NF-κB-binding sites in the genes for serum amyloid A and rat angiotensinogen (Fig. 10C) and to IL-1 and TNF-α regulatory regions of the human IL-6 genes (Fig. 10D). The AB region between positions 45 to 55 contains one sequence that is similar to the NF-κB-binding consensus sequence (37). A second NF-κB-like sequence is located at 59 to 69, overlapping the border between fragments B and C, and seems not to be significant for functionality of the DRE, as judged from fragment recombination analysis (Fig. 3). These NF-κB-like motifs within fragment B serve as nuclear protein-binding

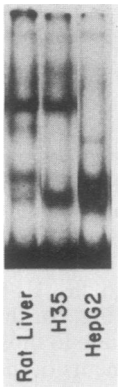

FIG. 9. DRE binding activities in the nuclear extracts from liver and hepatoma cells. Nuclear proteins were extracted from 4-h acute-phase liver and from H-35 and HepG2 cells treated for 13 h with IL-1 and IL-6. Equivalent amounts of nuclear proteins (1.5 μg) were included in binding reactions with end-labeled DRE. The complexes were separated on one gel. The autoradiogram was exposed for 24 h, and individual lanes were rearranged for better visual reproduction.

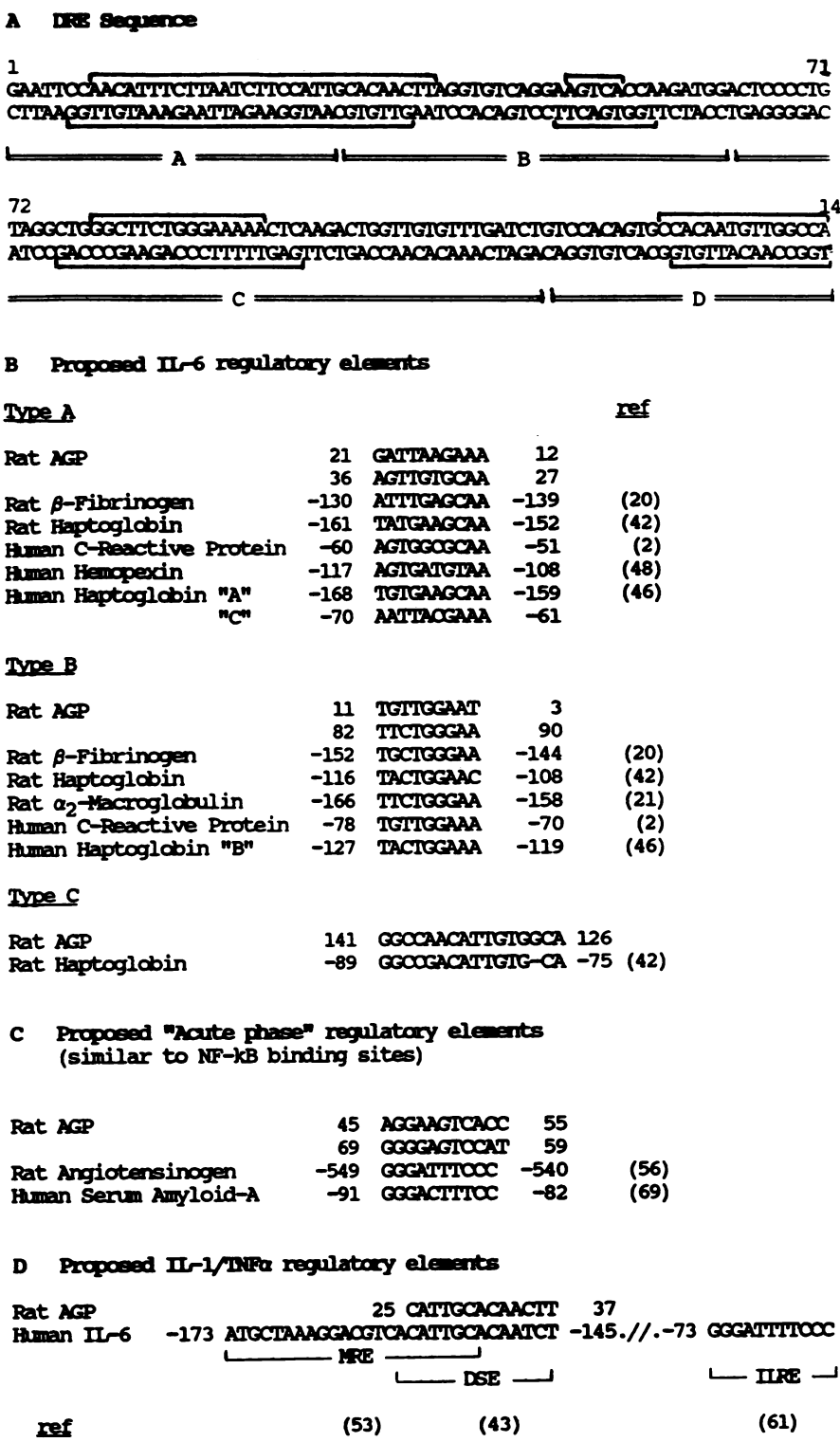

FIG. 10. Nucleotide sequence of the DRE (A) and comparison with sequences of other regulatory elements (B to D). The DRE nucleotide sequence CAT at positions 10 to 12 is a correction from the previously published sequence ACA (49). Brackets above and below the nucleotide sequence indicate DNase I-protected regions. Results from the analysis of the lower strand are not presented in this report. Double-lined brackets below indicate segments A to D of the DRE.

sites (Fig. 6B). Overlapping with this binding site is the sequence GGAAGTCA (46 to 53), which is similar to the 62-kilodalton protein-binding site GGATGTCC found in the serum response element of mouse c-*fos* (58) or GGACGTCA in the human IL-6 gene (53). However, this DRE motif does not confer any cytokine or TPA regulation to a heterologous promoter when tested alone. The lack of a TPA response indicates that the sequence does not function like the NF-κB

(or NF-κB-like)-binding site of the serum amyloid A gene (18), the angiotensinogen gene (56), or the promoter-proximal inflammatory lymphokine responsive element in the human IL-6 gene (61). However, the data in Fig. 3 demonstrate that the motif in DRE fragment B appears to be necessary in addition to the adjacent 5′ region A for reconstituting an IL-1 response element.

The major protein-binding site within AB (positions 8 to 37) is composed of several sequence motifs that have been implicated in cytokine responses of other genes. As already mentioned above, this region carries an inverted copy of type B and type A in tandem at the 5′ end, and at the 3′ end there is an additional inverted copy of the type A IL-6 regulatory element (Fig. 10B). The segment in the center (positions 25 to 37) is remarkably similar to the dyad symmetry element (43) located in the 3′ half of the multiple response element of the human IL-6 gene as defined by Ray et al. (53). This multiple response element mediates the transcriptional stimulation of the IL-6 gene by IL-1, TNF-α, phorbol ester, forskolin, and serum. Mutation of the sequence CGTCA which flanks the DSE within the multiple response element of the IL-6 gene (not present in the DRE) has led to reduced responsiveness to phorbol ester and forskolin but not to the other factors (53), suggesting that the DSE might be crucial for IL-1 and TNF-α regulation. Unlike the findings with the elements of the genes for IL-6, angiotensinogen, and serum amyloid A, the DRE sequences gain regulatory function only when the DSE-like motif is combined with the NF-κB-like motif (Fig. 3, constructs 21 and 22 versus constructs 5 and 6). We do not know which of the motifs within AB is specifically responsible for the TPA response and which are responsible for the IL-1 response. Separate targets are suggested by the additive action of the two factors (Table 2).

Although the functionally defined DRE regions serve as binding sites for nuclear proteins, we could not observe any cytokine-specific changes in the qualitative or quantitative pattern of DRE-binding activities (Fig. 6 to 8). This is in contrast to the reports of an IL-6-inducible protein in Hep3B cells which binds to the A and C domains of the IL-6 regulatory element of the human haptoglobin gene (45) and of an acute-phase-inducible protein in rat liver which binds to the IL-6 regulatory element of the rat α_2-macroglobulin gene (26). We consider two possible explanations for our results. The first is that the experimental techniques used are not sensitive enough to detect a minor species of a physically distinct binding protein in the pool of abundant, generic DRE-binding proteins. The titration of nuclear extract from hepatic cells (Fig. 7 and 8) or from nonhepatic cells (data not shown) indicated a relatively high concentration of DRE-binding activities. A more definitive search for cytokine-specific binding proteins will require chromatographic separation of nuclear extracts before binding analysis. Ongoing experiments should clarify this point. The second possibility is that the DRE is not a substrate for an inducible binding protein, but that the cytokines exert their influence through posttranslational modification of the constitutively DRE-bound proteins. Precedence for such a mechanism is found in the phosphorylation-dephosphorylation of transcription-controlling factors (13, 29, 51, 64, 70). However, a link between cytokine-regulated changes in specific kinase activities (12, 38) and changes in phosphorylation states of transcription factors remains to be documented.

The regulatory phenotype of a given gene element in a given cell type is dependent not only on the modular arrangement of the *cis*-acting DNA sequences but also on the relative composition of nuclear DNA-binding proteins or *trans*-acting factors. Therefore, it is not surprising to find quantitative differences in the cytokine regulation of the rat DRE in HepG2 and H-35 cells (Fig. 4 and 5) in view of the differences in DRE-binding proteins (Fig. 9). Nevertheless, a remarkably high conservation of regulatory specificity is maintained between cell types and species. A future task will be to identify the cellular and nuclear components that interact with the DRE and are involved in transcription control.

The regulation of plasma protein genes during an acute-phase reaction has been ascribed to the action of a variety of humoral factors on liver cells (5, 8). Principal roles are fulfilled by IL-1, TNF-α, IL-6, LIF, and glucocorticoids, but modulating influences by tumor growth factor β, insulin, glucagon, catecholamines, and prostaglandins cannot be ruled out. The combination of these influences contributes to the definition of acute-phase regulation. It seems unlikely that there is a single *cis*-acting element near an acute-phase plasma protein gene that is responsive to all signals that converge onto the gene. Indeed, the characterization of several acute-phase genes has revealed separate as well as overlapping *cis*-acting elements for IL-1, IL-6, or glucocorticoids (6, 42, 49). Each element in itself is again composed of several interacting regulatory sequences (40, 42, 45, 48). Undoubtedly, new elements and different arrangements of their functional sequences will be uncovered. Therefore, a precise nomenclature as well as a functional categorization of elements will be needed. A term such as "acute-phase element" (56) appears inappropriate; a more precise description would be achieved by addressing the primary regulatory hormone involved, i.e., IL-1, IL-6, TNF-α, LIF, or glucocorticoid response element or units (10, 59). Only the combined action of all of these elements will explain the overall phenomenon of acute-phase regulation.

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