Regulation of C4b-Binding Protein Gene Expression by the Acute-Phase Mediators Tumor Necrosis Factor-α, Interleukin-6, and Interleukin-1[†,‡]

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ABSTRACT: C4b-binding protein (C4BP) is involved in the fluid-phase regulation of the classical pathway of complement. During an acute-phase response, we have shown that hepatic levels of murine C4BP mRNA are elevated 2.5-fold while rat liver C4BP gene expression exhibits a 4-fold induction. Furthermore, a survey of different mouse tissues showed that during acute inflammation C4BP gene expression was confined to the liver. To gain a better understanding of the acute-phase regulation of C4BP gene expression we utilized the rat hepatoma cell line FAO in which tumor necrosis factor-α (TNF-α) produced a 2.7-fold induction of C4BP mRNA levels. In the absence of TNF-α, interleukin-1α (IL-1α) and interleukin-6 (IL-6) had little effect on C4BP gene expression but when all three cytokines were used together a synergistic 4-fold induction of C4BP mRNA levels was observed. In contrast the synthetic glucocorticoid dexamethasone inhibited TNF-α-induced C4BP gene expression. Cycloheximide-mediated inhibition of inducible C4BP gene expression demonstrated the requirement for ongoing protein synthesis. Rapid induction of C4BP mRNA levels by TNF-α and IL-6 (within 1 h) and the observation that stimulation was inhibited by actinomycin D provided evidence that regulation of C4BP gene expression during the acute-phase response is regulated at the transcriptional level. Isolation of a genomic clone extending into the 5′ regulatory region of the rat C4BP gene enabled us to identify the major transcriptional start site and putative response elements through which TNF-α, IL-6, IL-1α, and dexamethasone may exert their effects on C4BP gene expression. A structural comparison of the rat C4BP gene 5′ flanking sequence with the promoters of both the mouse and human C4BP genes revealed two 200 bp regions of striking homology. Further investigation is required to determine the functional relevance of these structural homologies.

Following the activation of immediate local responses, mammalian systemic tissue injury leads to an increase in the plasma concentration of a set of proteins known as the acute phase reactants [for review see Baumann (1989), Baumann and Gauldie (1990), and Fey and Fuller (1987)]. The liver serves as the primary site of synthesis of most of these proteins (Koj, 1974). No individual cytokine can elicit an entire acute phase response but interleukin-6 (IL-6), interleukin-1 (IL-1), and tumor necrosis factor-α (TNF-α) are the cytokines which when used alone or in combinations (and occasionally in the required presence of glucocorticoids) are able to stimulate distinct subsets of the major acute-phase proteins (Baumann et al., 1987; Darlington et al., 1986; Goldman & Liu, 1987; Perlmutter et al., 1986).

The most important function of the acute-phase response is to limit the harmful effects of tissue injury. Such protective effects are accomplished by, for example, inhibiting proteases or helping to modulate the immune response and contributing to blood clotting (Koj, 1987). Regardless of the cause of inflammation, e.g. infection, burn, or neoplastic growth, a typical pattern of qualitative and quantitative changes in acute-phase protein plasma concentration is observed. While the circulating levels of some reactants increase only 2–3-fold (e.g. complement C3 and α_1-macroglobulin) others are increased 100–1000-fold (e.g. serum amyloid P, α_2-macroglobulin, and C-reactive protein). In addition negative acute-phase reactants, e.g. albumin, display a transient decrease in synthesis rate (Birch & Schreiber, 1986; Kushner, 1982).

Recently the complement regulatory protein C4b-binding protein (C4BP) was identified as an acute-phase reactant in humans (Barnum & Dahlbäck, 1990; Saeki et al., 1989). C4BP can cause accelerated decay of the C3 convertase (C4b2a) by binding to C4b and displacing C2a and acts as a cofactor in the factor I-mediated cleavage of C4b. In addition C4BP can also bind to fluid phase C4b thus preventing formation of the C3 convertase (Burge et al., 1981; Fujita et al., 1978; Fujita & Nussenzweig, 1979; Gigli et al., 1979; Nagasawa et al., 1980). Therefore due to the role played by C4BP in regulating complement activation, a better understanding of cytokine-modulated C4BP gene expression will provide clues to regulatory mechanisms involved in controlling the acute-phase response.

EXPERIMENTAL PROCEDURES

Enzymes and Reagents

RNA isolation reagents, modifying enzymes, and restriction endonucleases were purchased from Bethesda Research Laboratories, Gaithersberg, MD, Promega Biotec, Madison, WI, New England Biolabs, Beverly, MA, and Boehringer Mannheim Biochemicals, Indianapolis, IN. Radiolabeled nucleotides were purchased from Amersham, Arlington Heights,IL.

In Vivo Acute-Phase Response

(*a*) *Murine.* Acute inflammation was induced in 10-week-old female CBA/J mice (Jackson Laboratories, Bar Harbor,

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ME) by ip injection of 1 mL of 3% (w/v) thioglycolate medium (Sigma, St. Louis, MO). At defined time intervals (0, 1, 2, 4, 8, 12, 18, 24, 48, and 72 h) following injection, two animals were killed by cervical dislocation, and various organs (liver, kidney, spleen, and lung) were removed and immediately frozen in liquid nitrogen. Total RNA was prepared from these tissues and examined by Northern blot analysis as previously described (Moffat et al., 1989).

(*b*) *Rat.* The RNA samples used for this experiment were kindly provided by Dr. Georg Fey, La Jolla, CA. An acute-phase response had been elicited in 10–20-week-old male Fisher 344 rats (Simonsen Laboratories, Gilroy, CA) by ip injection of 0.35 mL of complete Freund's adjuvant. Rats were killed at time 0 and at 18 h following injection, and their livers were excised and total cellular RNA prepared by a modified guanidinium hydrochloride procedure (Gehring et al., 1987). Levels of C4BP mRNA were determined by dot-blot hybridization (Moffat et al., 1989).

Cell Culture

The rat hepatoma cell line FAO (kindly provided by Dr. Georg Fey) was cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium with 4.5 g/L glucose (Whittaker Bioproducts, Walkersville, MD) and F12 nutrient mixture (Ham) with L-glutamine (Gibco Laboratories, Grand Island, NY) containing 10% fetal bovine serum (Upstate Biotechnology, Lake Placid, NY) and 1% penicillin/streptomycin/fungizone mixture (Whittaker).

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from murine tissue by the guanidinium thiocyanate method (Chirgwin et al., 1979) and from FAO cells by the acid guanidinium thiocyanate/phenol/chloroform extraction procedure (Chomczynski & Saachi, 1987). RNA (10 μg/lane) was electrophoretically fractionated and transferred to Hybond-N nylon membranes (Amersham) as described previously (Moffat et al., 1989). cDNA probes [a 927 bp PstI fragment (nucleotides 301–1228) of mouse C4BP, a 630 bp PvuII/SspI fragment (nucleotides 127–757) of murine serum amyloid P (Ishikawa et al., 1987; a gift from Dr. Naoki Maruyama, Tokyo, Japan), and an 800 bp EcoRI/HindIII fragment of chicken β-actin] were labeled by the random primed method (Feinberg & Vogelstein, 1983). All Northern blots were washed to high stringency: 0.1 × SSC containing 0.1% SDS at 65 °C for 30 min. The nylon membranes were stripped for rehybridization by incubation in a boiling solution of 0.1% SDS for 20 min. Autoradiography was performed using X-Omat AR film (Kodak) and two intensifying screens (Du Pont) at −70 °C for the appropriate time. Results were quantitated by laser densitometry using an LKB UltroScan XL densitometer.

Screening of the Rat Genomic Library

A rat liver genomic library constructed in the lambdaDASH vector (Stratagene, La Jolla, CA) was screened with a mouse C4BP 5′ PstI cDNA probe (nucleotides, 1–301) to generate the λrC4BPA and λrC4BPB clones. Both clones were plaque-purified and the DNA was isolated.

Sequencing of the Rat C4BP Promoter

DNA fragments of 300–600 bp in length were randomly generated by sonication and ligated into the SmaI site of M13mp8 after end-repairing with T4 DNA polymerase. Rat C4BP 5′ sequence-containing phage were identified by transferring a portion of the phage to nylon filters (MSI, Westboro, MA) in duplicate and probing the filters with random primed labeled mouse C4BP 5′ PstI cDNA probe (nucleotides 1–301). Filters were prehybridized and hybridized at 42 °C in 50% formamide buffer. Phage that screened positive were then sequenced by the dideoxy chain-termination method (Sanger et al., 1977) with [α-^{35}S]deoxyadenosine 5′-thiotriphosphate (Biggin et al., 1983) using the Sequenase kit (United States Biochemical, Cleveland, OH).

Primer Extension Analysis

From alignment with the mouse C4BP cDNA sequence (Kristensen et al., 1987), a 30-mer oligonucleotide 5′-GTCGACCCACGCGCAGGTGATCCTTGCATT-3′ complementary to nucleotides 31–60 of the rat C4BP cDNA sequence was end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase to a specific activity of 10^8 cpm/μg. The labeled oligo (5 ng) and 25 μg of RNA isolated from FAO cells were coprecipitated, resuspended in 30 μL of hybridization buffer (1 M NaCl, 1 M PIPES pH 6.4, and 25 mM EDTA, pH 8.0), denatured at 65 °C for 10 min, and annealed at 37 °C for 16 h.

The hybrids were resuspended in 45 μL of extension buffer (50 mM Tris HCl pH 8.2, 6 mM $MgCl_2$, 10 mM DTT, 100 mM NaCl, 0.33 mM dNTPs, 40 units RNasin), and the primer was extended using 50 units of AMV reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 42 °C for 2 h. Following RNase A digestion and phenol/chloroform extraction, the extended products were analyzed on a 7% denaturing polyacrylamide gel with a sequenced M13 DNA template from the same region as a size marker.

RESULTS

Identification of C4BP as an Acute-Phase Reactant. In vitro and in vivo studies have identified human C4BP as an acute-phase reactant (Barnum and Dahlbäck, 1990; Saeki et al., 1989). To determine whether C4BP plays a similar role in mice, an acute-phase response was induced in 10-week-old female CBA/J mice by ip injection of 3% thioglycolate medium (1 mL). Two animals were killed at each time point (0, 1, 2, 4, 8, 12, 18, 24, 48, and 72 h) following injection, and hepatic levels of C4BP mRNA were measured (Figure 1). A 2.5-fold increase in C4BP mRNA levels was detected 8 h following injection and remained elevated before returning to basal expression at 48 h. This modest induction is consistent with the observed 2–3-fold stimulation of C4BP in humans during acute inflammation (Barnum & Dahlbäck, 1990; Saeki et al., 1989). Conclusive evidence that an acute-phase response had been induced by the thioglycolate medium was provided by the observed stimulated expression of the major acute phase reactant, serum amyloid P. Furthermore, the 5-fold elevation of serum amyloid P mRNA levels, although more pronounced, exhibited similar kinetics of C4BP induction.

We have previously shown that murine C4BP gene expression is largely confined to the liver (Moffat et al., 1992). To follow the tissue distribution of C4BP mRNA during acute inflammation total RNA was isolated from various organs (kidney, spleen, and lung) removed at different times (0, 8, and 24 h) following induction of the acute-phase response and examined for C4BP mRNA levels (Figure 2). C4BP gene expression was detectable in liver but not kidney, spleen, or lung and therefore this experiment clearly shows that during an inflammatory response C4BP and mRNA production is still governed by a strict regime of liver-enriched gene expression.

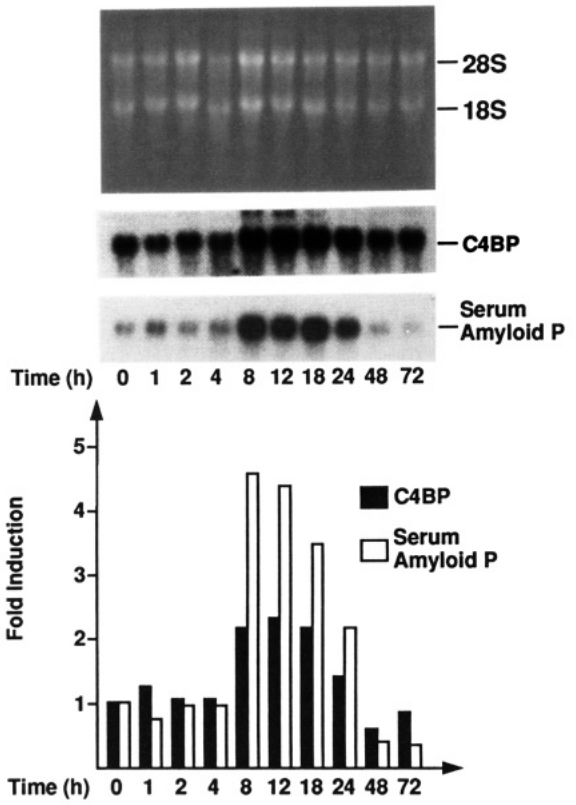

FIGURE 1: An acute-phase response was induced in 10-week-old female CBA/J mice by ip injection of 1 mL thioglycolate medium (3% w/v). At defined time intervals following injection the animals' livers were removed, and total RNA was isolated and analyzed sequentially by Northern blot analysis (5 μg/lane) for C4BP and serum amyloid P mRNA levels. Quantitation was performed by laser densitometry, and values were corrected for levels of actin mRNA (data not shown). Representative samples from one of the two animals sacrificed at each time point are shown. Detection of a minor 3.0 kb C4BP transcript provides evidence for the existence of a low abundance precursor C4BP mRNA species in mouse liver.

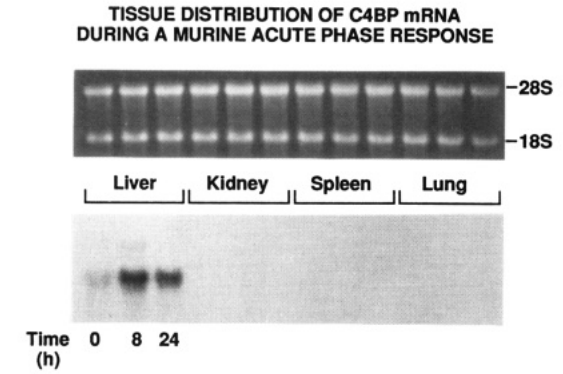

FIGURE 2: Tissue distribution of C4BP mRNA during a murine acute-phase response. Various organs (kidney lung and spleen) were removed from the animals described in Figure 1, and total RNA was isolated and analyzed for the presence of C4BP mRNA (10 μg/lane). The ethidium bromide-stained Northern gel is shown to demonstrate equal loading of the samples.

A more detailed examination of the molecular regulation of murine C4BP gene expression during acute inflammation required the identification of a suitable model cell line. However the two best documented mouse hepatoma cell lines, Hepa (Darlington et al., 1980) and BW (Szpirer & Szpirer, 1975) have not been well-characterized with respect to the acute-phase response. In contrast a number of detailed studies of acute inflammation have been performed in rat hepatoma cell lines—FAO, H5, FAZA (Deschatrette & Weiss, 1974), Fto2B (Killary & Fournier, 1984), H35 (Baumann et al., 1987) and HTC.JZ1 (Vannice et al., 1983). Therefore due to the availability of these cell lines, a study of rat C4BP proved to be a more practical approach for an in-depth examination of acute-phase C4BP gene regulation. In this regard, Figure 3 demonstrates that 18 h following an experimentally-induced acute-phase response C4BP mRNA levels were elevated 4-fold in rat liver.

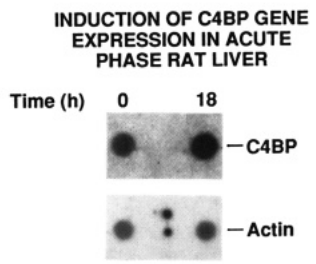

FIGURE 3: An experimentally-induced acute-phase response was elicited in 10–20-week-old male Fisher 344 rats by ip injection of 350 μL of complete Freund's adjuvant. Total RNA was isolated at time 0 and 18 h following injection and applied to a Hybond-N nylon membrane (5 μg RNA/dot). Levels of C4BP mRNA were quantitated using an LKB UltroScan XL densitometer and normalized for the level of actin gene expression.

To identify a suitable in vitro model for studying the acute-phase regulation of C4BP gene expression, we surveyed a number of rat hepatoma cell lines—FAO, HTC.JZ1, H5, FAZA, and Fto2B (generously donated by Dr. Georg Fey). The differentiated FAO cell line was selected for these studies by virtue of its modest basal levels of C4BP gene expression that were significantly inducible by the addition of the acute-phase mediators—TNF-α, IL-6, and IL-1α. In addition FAO cells retain many functional properties of primary rat hepatocytes and have therefore been shown to be a suitable model for the study of hepatic gene expression (McCracken et al., 1984).

Optimal Cytokine Regulation of C4BP Gene Expression. Initial experiments were designed to identify which cytokine or combination of cytokines produced the maximum effects on C4BP gene expression in FAO cells. The results of these studies are presented in Figure 4 [preliminary experiments determined the optimal cytokine and glucocorticoid concentrations (data not shown)]. Levels of actin gene expression were used to standardize the results, and the corrected values of induction are presented in the histogram shown in Figure 4. Clearly, TNF-α (500 units/mL) had the major stimulatory effect and induced C4BP gene expression 2.7-fold above basal levels (lane 4). In fact IL-6 or IL-1 either alone or together did not produce any significant effects on C4BP gene expression. However the addition of all three cytokines, IL-1α (200 units/mL), IL-6 (500 units/mL), and TNF-α, resulted in maximal 4-fold stimulation of C4BP gene expression (lane 12).

Interestingly the presence of the glucocorticoid dexamethasone (Dex; 1 μM) completely inhibited TNF-α-induced stimulation of C4BP mRNA levels to below basal levels of expression (compare lanes 4 and 11). However IL-1α (lane 14) and to a greater extent IL-6 (lane 15) served to limit the inhibitory effects of Dex on TNF-α-induced C4BP gene expression.

Kinetics of Cytokine-Mediated Induction of C4BP Gene Expression. FAO cells were incubated with IL-6 and TNF-α and at various time points total cellular RNA was prepared. Northern blot analysis was then performed to follow the appearance of elevated C4BP mRNA levels (Figure 5). These results clearly show that the induction of C4BP gene expression by IL-6 and TNF-α exhibited no substantial lag period and

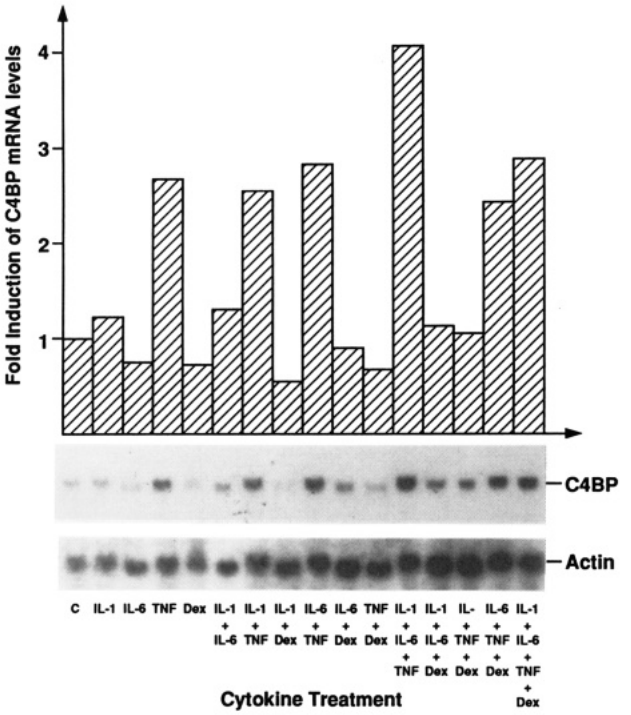

FIGURE 4: Confluent monolayers of FAO cells (2.5×10^6 cells/plate) were treated with combinations of TNF-α (500 units/mL), IL-6 (500 units/mL), IL-1α (200 units/mL) and Dex (1 μM) for 18 h. Following this treatment, total RNA was isolated from the cells and examined by Northern blot analysis (10 μg/lane). Levels of C4BP mRNA were detected using a mouse C4BP PstI (927 bp) cDNA probe and quantitated using an LKB UltroScan XL densitometer. Values were corrected for the level of actin mRNA, and the results are presented in a histogram. This study was performed in duplicate, and representative samples from one experiment are shown.

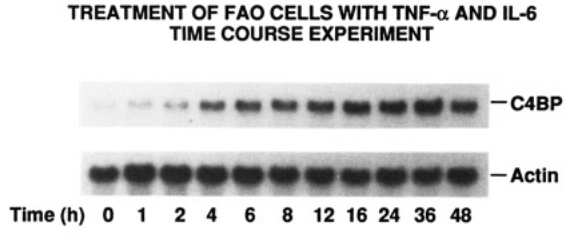

FIGURE 5: Confluent monolayers of FAO cells (10^7 cells/plate) were treated with a combination of TNF-α (500 units/mL) and IL-6 (500 units/mL). At various time points total RNA was isolated and examined by Northern blot analysis (10 μg/lane). Levels of C4BP mRNA were determined and standarized using a β-actin cDNA probe.

levels began to increase within 1 h of treatment. C4BP mRNA levels peaked at 16 h and remained elevated before beginning a return to basal levels of expression after 48 h. Levels of actin mRNA are shown to demonstrate equal loading of each RNA sample.

Acute-Phase Regulation of C4BP Gene Expression Is Protein Synthesis Dependent. The rapid induction of C4BP gene expression by IL-6 and TNF-α led us to believe that regulation was mediated by preexisting intracellular control mechanisms. However the addition of cycloheximide (10 μg/mL), a protein synthesis inhibitor, completely abolished the stimulation of C4BP mRNA levels (Figure 6). The inhibitory effect was identical whether cycloheximide was added at the same time as TNF-α and IL-6 or either 1 h before or after cytokine treatment. Furthermore when cycloheximide was used alone, C4BP mRNA was no longer detectable and we

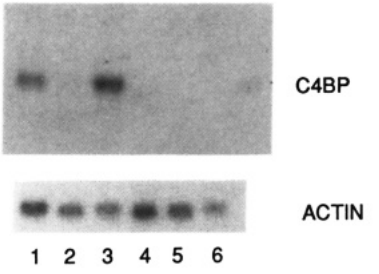

FIGURE 6: Treatment of FAO cells with IL-6 and TNFα and effect of cycloheximide (10 μg/mL) on C4BP mRNA levels. Confluent monolayers of FAO cells (2.5×10^6 cells/plate) were treated with a combination of TNF-α (500 units/mL) and IL-6 (500 units/mL) in the presence or absence of cycloheximide (CHX; 10 μg/mL) for 18 h. Total RNA was then isolated and examined by Northern blot analysis (10 μg/lane). Lane 1, control; lane 2, CHX alone; lane 3, TNF-α/IL-6 alone; lane 4, simultaneous treatment with CHX and TNF-α/IL-6; lane 5, cells pretreated for 1 h with CHX before addition of TNF-α/IL-6; lane 6, cells pretreated for 1 h with TNF-α/IL-6 before addition of CHX.

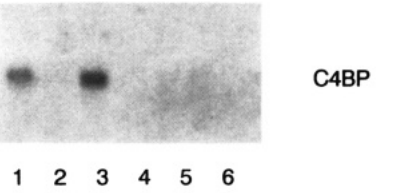

FIGURE 7: Treatment of FAO cells with IL-6 and TNFα and effect of actinomycin D (10 μg/mL) on C4BP mRNA levels. Confluent monolayers of FAO cells (2.5×10^6 cells/plate) were treated with a combination of TNF-α (500 units/mL) and IL-6 (500 units/mL) in the presence or absence of actinomycin D (ActD; 10 μg/mL) for 18 h. Total RNA was then isolated and examined by Northern blot analysis (10 μg/lane). Lane 1, control; lane 2, ActD alone; lane 3, TNF-α/IL-6 alone; lane 4, simultaneous treatment with ActD and TNF-α/IL-6; lane 5, cells pretreated for 1 h with ActD before addition of TNF-α/IL-6; lane 6, cells pretreated for 1 h with TNF-α/IL-6 before addition of ActD.

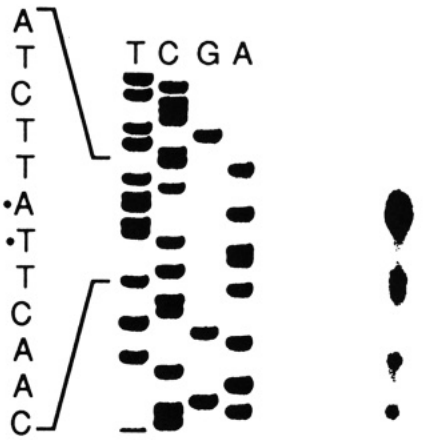

FIGURE 8: Primer extension analysis of rat C4BP mRNA. A 30-mer (5 ng) complementary to the rat C4BP cDNA sequence was end-labeled, annealed to 25 μg of total RNA isolated from FAO cells, and extended using AMV reverse transcriptase. An M13 template containing sequence from the same origin was run as a size marker. The extended products align with the A and T residues marked (•) as transcription start sites. However the stronger intensity of the band aligning with the T residue led us to assume that this nucleotide represented the major transcription start site.

can therefore conclude that in FAO cells basal and inducible C4BP gene expression requires de novo protein synthesis.

Evidence That Regulation of Cytokine-Inducible C4BP Gene Expression Occurs at the Transcriptional Level. Stimulation of C4BP mRNA levels by TNF-α and IL-6 may be controlled transcriptionally and/or by post-transcriptional mechanisms.

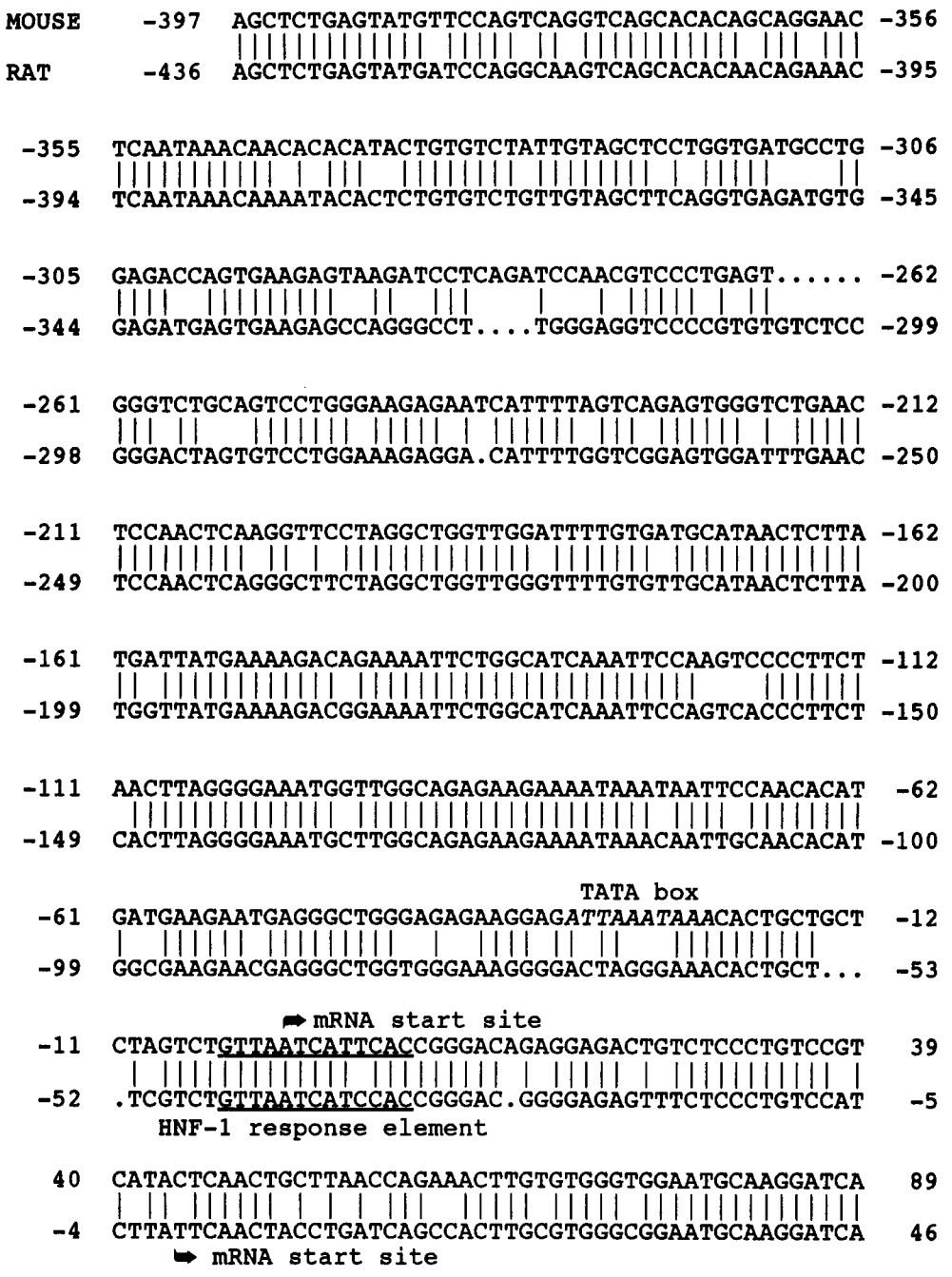

FIGURE 9: Homology between the 5′ regulatory regions of the mouse and rat C4BP genes. Sequence analysis demonstrates a high degree of homology (85%) that extends at least 950 bp upstream. However unlike the mouse gene there is no putative TATA box in the promoter region of the rat C4BP gene. The underlined region denotes the strongly conserved HNF-1 binding site.

As an initial approach to understanding this mechanism we examined the effects of a transcription inhibitor, actinomycin D, on the cytokine-mediated induction of C4BP gene expression. Figure 7 shows that in the presence of actinomycin D (10 μg/mL), TNF-α and IL-6 lost the ability to increase C4BP mRNA levels. An identical inhibitory effect was observed when the cells were treated simultaneously with TNF-α, IL-6, and actinomycin D or pretreated for 1 h with either actinomycin D or the two cytokines. These results therefore imply that regulation of C4BP gene expression by TNF-α and IL-6 occurs at the transcriptional level.

Isolation of a Rat C4BP-Promoter-Containing Genomic Clone and Its Use in the Identification of the C4BP Gene Transcriptional Start Site. To begin an investigation of the molecular events that control the acute-phase elevation of C4BP gene expression it was necessary to isolate a genomic clone that contained the rat C4BP promoter. Using a probe that spanned the first 300 bp of the mouse C4BP cDNA sequence (Kristensen et al., 1987), we screened a rat liver genomic library and isolated two genomic clones, λrC4BPA and λrC4BPB. The orientation and position of sequences derived from these clones was determined by alignment with the homologous murine C4BP genomic sequence (Moffat et al., 1992). Primer extension analysis was then performed to precisely map the transcriptional start site of the rat C4BP gene (Figure 8). The major transcription start site was found to be the T residue located 42 bp downstream relative to the murine C4BP transcription start site (for comparison of sequences see Figure 9). Repetitive primer extension experiments consistently gave the same results as shown in Figure 8 where the reverse transcriptase has clearly terminated elongation at several residues prior to the major transcription

Table I: Putative Response Elements in the Rat C4BP Enhancer/Promoter[a]

HNF-1 response element	GTTAATNATTAAC
	-46 GTTAATCATccAC -34
NF-κB response element	GGGGACTTTCC
	-309 GGGGACcTcCC -319
AP-1 response element	TGAGTCA
	-427 TGAGTCt -433
	-340 TGAGTgA -334
IL-6 response element	CTGGGA
	-720 CTGGGgA -714
	-322 CTtGGGA -316
	-81 gTGGGA -76
	-68 CTaGGGA -62
NF-IL-6 (LAP) response element	ACATTGCACAATCT
	-530 ACATTaGCAtctTCaT -515
	-216 gtgTTGCAtAAcTCT -202
	-114 ACAaTTGCAaCAcATgg -98
glucocorticoid response element	GGTACANNNBGTYCT
	-849 GGTACA(N)AGTTCT -836

[a] N, any base; B, A or T; Y, C or T; lower case letters denote nonhomologous bases.

start site. However the relative intensity of these bands is low and therefore may represent minor transcription start sites or perhaps artifacts caused by RNA secondary structure-induced abrogation of reverse transcriptase activity. Identical results were obtained with RNA isolated from either untreated FAO cells or cells stimulated with TNF-α and IL-6 (data not shown) demonstrating that the same transcription start site is utilized for both basal and cytokine-induced C4BP gene expression. Unlike the mouse gene this transcription start site was not associated with putative TATA and CAAT boxes. However lack of these eukaryotic promoter elements was also recently reported for the human C4BP gene (Rodriguez de Cordoba et al., 1991).

Similar to findings in the 5′-untranslated sequence of both the murine and human C4BP genes (Moffat et al., 1992; Rodriguez de Cordoba et al., 1991), two upstream AUG codons (positions 35 and 119) are found in the rat C4BP gene. Both of these initiation triplets are associated with in-frame termination codons and therefore may give rise to upstream open reading frames.

Identification of Putative Response Elements. Our results have clearly demonstrated that acute-phase mediators, most notably TNF-α, are able to elevate C4BP mRNA levels in FAO cells. In addition the rapid induction of C4BP gene expression by IL-6 and TNF-α and the ability of actinomycin D to prevent induction suggest that regulation occurs at the transcriptional level. Following the isolation of the two genomic clones described above, we analyzed the rat C4BP promoter for the presence of putative cis-acting elements through which the cytokines may exert their effects on C4BP gene expression. The results of this computer-aided analysis are shown in Table I. TNF-α has been shown to regulate gene expression via a number of transcription factors but predominantly operates through NF-κB (Duh et al., 1989; Lowenthal et al., 1989; Osborn et al., 1989; Zhang et al., 1990) and AP-1 (Brenner et al., 1989). The rat C4BP promoter displayed one good match with an NF-κB response element (Sen & Baltimore, 1986) and two regions of homology with the AP-1 binding site (Angel et al., 1987). IL-6 has been shown to exert its effects on the rat α_2-macroglobulin gene via a CTGGGA motif (Hattori et al., 1990). Four elements displaying homology to this sequence were identified. As well as stimulating NF-κB activity (Lenardo & Baltimore, 1989), IL-1 can also induce gene expression via the NF-IL-6 transcription factor (Akira et al., 1990). LAP, the rat homologue of this factor, has recently been cloned (Descombes et al., 1990), and three elements homologous to the sequence bound by this protein were found in the rat C4BP promoter. One region exhibiting strong identity with a glucocorticoid response element (Evans, 1988) was identified as well as four additional half-sites. In addition to the elements described above we observed a sequence homologous to the binding motif for hepatocyte nuclear factor-1 (HNF-1), a liver-enriched transcription factor (Courtois et al., 1988). Therefore we have identified several candidate response elements that may play a role in the transcriptional regulation of C4BP gene expression by cytokines and glucocorticoids. The functional significance of these elements is currently under investigation.

Homology between the Rat, Mouse, and Human C4BP Promoters. Sequence derived from the rat genomic clones allowed a direct alignment and comparison to both the mouse and human C4BP promoters. As expected mouse and rat coding sequences displayed very strong homology (>85%) but remarkably this similarity was maintained for at least 950 bp upstream (Figure 9). However despite this strong degree of identity, the rat gene, unlike its mouse counterpart, is not associated with putative TATA and CAAT boxes. Moreover the location of putative response elements are in general not well-conserved between the two species, with the notable exception of the HNF-1 binding site.

Comparison of both sequences to the human C4BP promoter (Dr. S. Rodriguez de Cordoba—personal communication) revealed another interesting phenomenon. Although human C4BP exon 1 displays only modest homology with either mouse (46.3%) or rat (46.1%) exon 1, the promoters of these three genes display two distinct regions of strong homology (Figure 10). The 5′ proximal region which extends approximately 200 bp upstream of the HNF-1 site (highly conserved in all three species and used as a reference point for this comparison) exhibits the strongest identity (human:mouse = 74.6% and human:rat = 77.4%). The second homologous region is found a further 200 bp upstream and although not as conserved as the proximal region it still exhibits significantly stronger homology than its flanking sequences (human:mouse = 60.2% and human:rat = 61.2%).

DISCUSSION

These studies have demonstrated that during an acute-phase response murine liver C4BP mRNA levels are elevated 2.5-fold. However limitations imposed by the lack of a suitable mouse in vitro culture system led to a more detailed examination of the observed 4-fold induction of C4BP gene expression in acute-phase rat liver. In vitro experiments identified TNF-α as the principal effector of this response in the rat hepatoma cell line FAO. Furthermore this TNF-α mediated induction was augmented by the presence of both IL-1α and IL-6 resulting in an optimal 4-fold increase in C4BP mRNA levels.

Glucocorticoids have been shown to have a permissive and in some cases a synergistic effect on the in vitro induction of rat acute-phase reactants (Bauer et al., 1986; Baumann et al., 1983; Baumann et al., 1987; Koj et al., 1984). However when FAO cells are treated with TNF-α, Dex acts to prevent the stimulation of C4BP gene expression.

The mechanisms by which Dex exerts these effects are unknown. Our results have provided evidence that acute-phase regulation of C4BP gene expression occurs at the transcriptional level. Therefore one possible explanation for the observed Dex effects may be that inhibitory interactions of the relevant transcription factors may down-regulate the

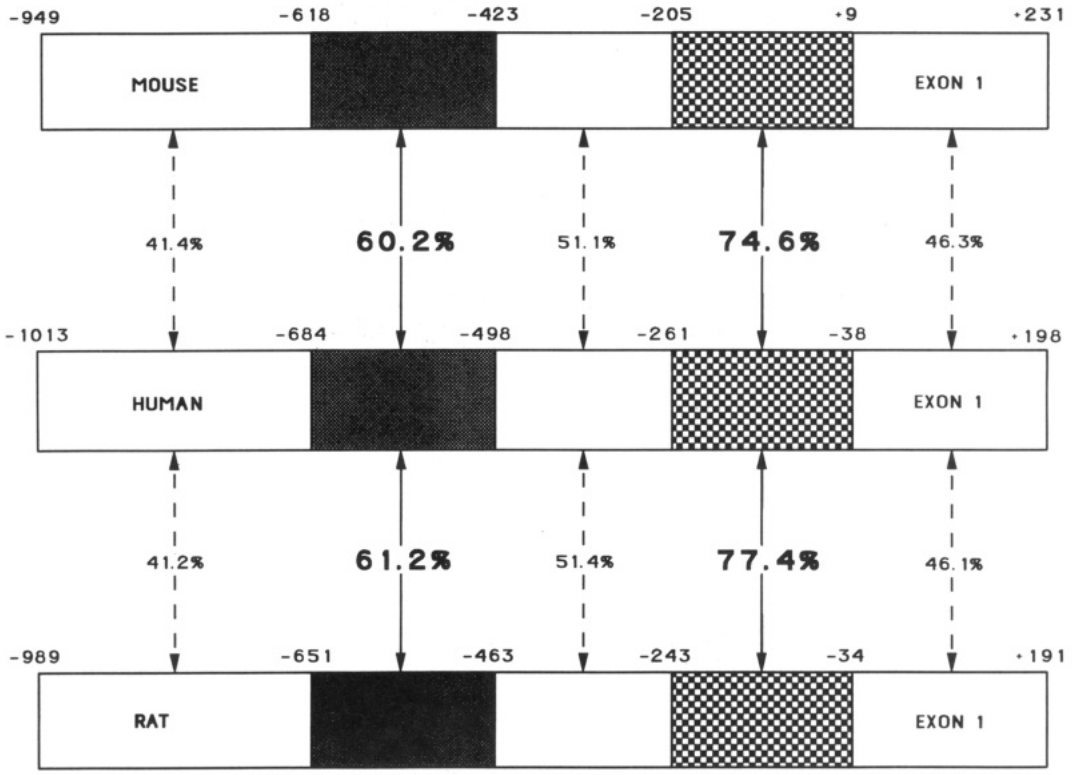

FIGURE 10: Highly conserved regions in the promoters of the mouse, human, and rat C4BP genes. Computer anaylsis identified the highlighted regions as areas of strong homology shared in all three species.

transcriptional activation capability of these proteins. TNF-α has been shown to stimulate transcription via the AP-1 (fos/jun heterodimer) transcription factor (Brenner et al., 1989). In addition several laboratories (Jonat et al., 1990; Schule et al., 1990; Yang-Yen et al., 1990) have demonstrated that the activated glucocorticoid-receptor complex can directly interact with AP-1 in a functionally antagonistic manner. This may be one mechanism by which Dex acts to diminish TNF-α-induced C4BP gene expression.

The protein synthesis-dependence of acute-phase C4BP induction was clearly demonstrated by the ability of cycloheximide to completely abolish C4BP gene expression. Interestingly, pretreatment of the cells for 1 h with TNF-α and IL-6 did not prevent the cycloheximide inhibitory effect although we had shown that these cytokines induced C4BP gene expression within 60 min. However we also demonstrated that cycloheximide not only inhibits the acute-phase induction of C4BP mRNA levels in FAO cells but also terminates basal C4BP gene expression. We can therefore conclude that ongoing protein synthesis is required for C4BP gene expression in FAO cells. Presumably the failure to detect C4BP gene expression following cytokine pretreatment is due to a short half-life of C4BP mRNA in these cells.

Rapid induction of C4BP gene expression by TNF-α and IL-6 and inhibition of this effect by actinomycin D argues that the response may be regulated at the level of transcription. These data, in light of the findings from the cycloheximide-treated cells, suggest that synthesis of a protein involved in the transcriptional regulation of the C4BP gene is required for expression of C4BP in FAO cells. Nuclear run-on assays and mRNA half-life measurements are currently being performed to investigate the regulatory mechanism in greater detail.

Potential upstream open reading frames have been identified in the 5′-untranslated sequence of the rat, mouse, and human C4BP genes. Upstream AUG triplets are present in less than 10% of vertebrate mRNAs and are most commonly found in genes involved in the regulation of cellular proliferation and differentiation e.g. proto-oncogenes (Kozak, 1987). Furthermore the existence of upstream open reading frames can produce dramatic effects on the translational efficiency of mRNAs (Kozak, 1989; Kozak, 1991). Therefore the role played by these elements in controlling translation of C4BP mRNA in all three species is an important issue that remains to be addressed.

A most intriguing aspect of these studies was the identification of highly conserved regions in the promoters of the rat, mouse, and human C4BP genes. The conservation is made all the more remarkable by the finding that the mouse gene is associated with consensus TATA and CAAT boxes (Moffat et al., 1992) which are absent in both the rat and human genes. Therefore a detailed comparison of the transcriptional activity of these three promoters will prove invaluable to ascertain the functional relevance of these structural homologies.

The identification of C4BP as an acute-phase reactant is important because of the role played by C4BP in regulating complement activation and therefore inflammation. Several other acute-phase proteins have been shown to have the ability to control immune response reactions [for review see (Koj, 1987)]. For example, the chemotactic responses of monocytes can be inhibited by the α-macroglobulins which also have the ability to modulate the responses of lymphocytes to mitogenic and antigenic stimuli (James, 1980). In addition many acute-phase proteins have been identified as protease inhibitors, e.g. α_1-proteinase inhibitor limits lung tissue damage during inflammation by the inhibition of leukocyte elastase activity (Fey & Fuller, 1987). In rats, thiostatins serve as potential sources of bradykinins and have been identified as cysteine proteinase inhibitors (Esnard & Gauthier, 1983). Moreover the cytolytic activity of natural killer cells and cytotoxic T-cells, which is in part conferred by serine proteases (Masson &

Tschopp, 1987), is inhibited by three acute-phase protease inhibitors—α_1-proteinase inhibitor, α_1-antichymotrypsin, and α_2-macroglobulin (Ades et al., 1982; Gravagne et al., 1982). These findings have particular relevance to C4BP which may also be defined as a serine protease inhibitor by virtue of its participation in the inhibition of the classical pathway C3 convertase, C4b2a. The C2 component is a novel type of serine protease (Kerr & Porter, 1978; Polley & Müller-Eberhard, 1968) which upon activation is responsible for the enzymatic cleavage of C3 (Cooper, 1975; Goldlust et al., 1974; Medicus et al., 1976). Therefore C4BP may be included in the group of acute-phase proteins which act to control the physiological effects of inflammation by the inhibition of serine protease activity.

By definition the acute-phase response is a rapidly induced systemic reaction to many different causes of inflammation. However the transient nature of this response is as striking as its magnitude and therefore stringent regulatory mechanisms must play a major role. C4BP, as an acute-phase reactant and inhibitor of complement activation, is most likely an integral component of this regulatory mechanism. Therefore a more complete understanding of the cytokine-induced regulation of C4BP gene expression may provide clues to the molecular mechanisms utilized to control the acute-phase response.

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