Cloning and tissue-specific expression of the gene for mouse C-reactive protein

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C-reactive protein is a serum acute-phase reactant that increases several thousand-fold in concentration during inflammation in most mammals. However, mouse C-reactive protein is considered to be a minor acute-phase reactant, since its blood level increases only from approx. 0.1 to 1–2 µg/ml. A mouse genomic clone of ~ 5 kb was obtained to determine the molecular basis for the regulation of the expression of mouse C-reactive protein. Several *cis*-acting elements in the 5' flanking region that potentially regulate transcription were identified: two glucocorticoid-responsive elements, two CCAAT-enhancer-binding protein C (C/EBP) consensus elements that are required for the interleukin-1 responsiveness of some acute-phase reactant genes, an interleukin-6-responsive element, two hepatocyte nuclear factor-1

(HNF-1) elements and a single heat-shock element. Transfection of the hepatoma cell line Hep 3B.2 with a pCAT expression vector containing the 5' flanking sequence from –1083 to –3 bp from the transcriptional start site, and truncations of this sequence, localized elements that control the tissue-specific expression of mouse C-reactive protein to the two HNF-1 elements and a C/EBP, interleukin-1-responsive element located between –220 and –153, and –90 and –50 bp from the transcriptional start site. A constitutive nuclear protein from mouse-liver hepatocytes specifically binds to the HNF-1 elements. These findings explain the tissue-specific expression of the gene, as well as its limited expression during the acute-phase response.

INTRODUCTION

C-reactive protein (CRP) is the prototypical serum acute-phase reactant (APR) in most mammalian species, and is synthesized by liver hepatocytes [1,2]. CRP is a member of the pentraxin family, and is composed of five identical, noncovalently linked subunits of ~ 23 kDa arranged in a flat pentameric disk with cyclical symmetry [2,3]. Although a unique function has not been ascribed to CRP, purified human CRP (huCRP) displays a variety of pro-inflammatory, host-protective activities, including complement activation, opsonization and leukocyte activation, which probably contribute to nonspecific host protection during the pre-immune phase of the inflammatory response (reviewed in [4–6]). Studies of CRP function *in vivo* have been limited by the lack of a suitable laboratory animal model. Mouse CRP (moCRP) is classified as a minor APR since its highest concentration was estimated at only ~ 2 µg/ml, as against levels of ≥ 300 µg/ml in humans that are undergoing an inflammatory response [1,6]. HuCRP and moCRP are each encoded by a single-copy gene on chromosome 1 of both species as part of a syntenic group of genes [7]. Both genes have a very similar organization, with two exons and a single intron within the codon for the third residue of the mature protein [8–10]. The amino acid sequences from cDNA clones for human and mouse CRP show an identity of 71% [10,11]. A few reports describe the isolation and detection of small amounts of moCRP by Ca²⁺-dependent binding to the definitive CRP substrate, phosphocholine [12–14], and cross-reactivity with huCRP [15,16]. Recently, we reported that interleukin-1 (IL-1), but not

IL-6, triggers the expression of the moCRP gene in isolated liver hepatocytes in culture [17]. By contrast, the most potent inducer of huCRP gene expression at the level of transcription is IL-6 [18–20]. The molecular basis of the control of the expression of the human and mouse CRP genes is not fully understood.

Gene expression is the result of the combined effects of a set of *trans*-acting factors interacting with multiple *cis*-acting elements [21]. Tissue-specific gene expression is regulated at the transcriptional initiation step through the recognition of promoter sites by transcription factors [22]. This general scheme for tissue-specific gene expression has been documented for many cell types, including pancreatic endocrine cells [23], hepatocytes [24], erythrocytes [25], muscle cells [26] and B cells [27,28]. For liver-specific gene expression, several distinct promoter and enhancer elements have been described, along with the corresponding liver-specific or liver-enriched DNA-binding proteins, including hepatocyte nuclear factor-1 (HNF-1) [29], CCAAT-enhancer-binding protein (C/EBP) [30], and D-region-binding protein, a transcriptional activator of the albumin gene [31]. HNF-1 recognizes the sequence GTTAATNATTAAC [29], which is present in the genes for albumin [32], pre-albumin [33], aldolase B [34], pyruvate kinase [35], α₁-antitrypsin [36], β-fibrinogen [36] and human CRP [37]. Among these genes, α₁-antitrypsin, β-fibrinogen and human CRP are considered to be positive APRs [38]. The C/EBP element is also found in the promoter regions of several hepatic genes, including huCRP [39], and the rat acute-phase genes for serum amyloid A1, α₁-acid glycoprotein, haptoglobin, and β-fibrinogen [40,41]. The C/EBP element is required for IL-1-induced human C3 gene expression

Abbreviations used: APR, acute-phase reactant; CRP, C-reactive protein; SAP, serum amyloid P-component; mo, mouse; hu, human; HNF-1, hepatocyte nuclear factor-1; C/EBP, CCAAT-enhancer-binding protein; CAT, chloramphenicol acetyltransferase; GRE, glucocorticoid-response element; HSE, heat-shock element; NF-IL6, nuclear factor for the IL-6 gene; H-ALB-PE, proximal element of the human albumin promoter; RE, responsive element; SP1, simian virus 40 promoter; NF1/CTF, nuclear factor-1/CCAAT-binding transcription factor; AP1, activator protein-1; SV40, simian virus 40; IL, interleukin.

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[42]. The increased production of most APRs is driven by a series of polypeptide factors, notably IL-1 and IL-6, that are released from cells at the inflammatory site, especially endothelial cells, fibroblasts and macrophages [38,43]. Endogenous mRNA levels for several mouse APRs, such as C3, serum amyloid P-component (SAP) and CRP, as well as several of the human and rat APR genes [41,43] are present at significant constitutive levels in hepatocytes [17,44]. It seems likely therefore that the expression of the mouse acute-phase genes is regulated by liver-specific *trans*-acting factors and *cis*-acting elements within the 5' flanking region of the genes. We report here the cloning of the moCRP gene, and the identification of two functional tissue-specific elements in the 5' regulatory region of the moCRP gene. This material has been previously presented in abstract form at the annual meeting of the American Society for Cell Biology, Abstr. 416, November 15–19 1992, Denver, CO, U.S.A.

MATERIALS AND METHODS

Cells and tissue culture

All cell lines were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The human hepatoma cell line Hep 3B.2 was grown in minimal essential Eagle medium containing 10% (v/v) fetal bovine serum, non-essential amino acids and sodium pyruvate. The human epithelial carcinoma cell line HeLa was cultured with 10% (v/v) defined calf bovine serum. All cells were monitored for mycoplasma infection using an enzyme-immunoassay kit and treated with BM-cycline (Boehringer–Mannheim, Indianapolis, IN, U.S.A.) for three passages to eliminate mycoplasmas, as described elsewhere [45].

Isolation of a genomic clone for moCRP

Liver DNA from C57BL/6 mice was isolated by the methods of Gross-Bellard et al. [46]. The genomic DNA was digested with *EcoRI* and separated by electrophoresis. The DNA fragments were transferred on to nylon membranes (Nytran, Schleicher and Schuell, Inc.) and hybridized with cDNA for mouse CRP (MCRP1) [11] that had been random-labelled with [α - 32 P]dCTP (Amersham) using an oligolabelling kit (Pharmacia, Piscataway, NJ, U.S.A.). Hybridization and washing were performed by the methods of Maniatis et al. [47]. An *EcoRI* fragment of approx. 5 kb that hybridized with the cDNA probe in Southern blots was observed [10]. Size-selected DNA (4.5–6 kb) was ligated to *EcoRI*-digested λ ZapII bacteriophage arms, and packaged using Gigapack Gold extracts (Stratagene, La Jolla, CA, U.S.A.). The recombinant phages were grown as plaques in *Escherichia coli* strain XL-1 Blue (Stratagene). The screening procedure used the phage library diluted to about 1.5×10^4 plaques/100 mm plate. After 12–16 h the plaques were lifted from the agarose overlay on to nylon membranes (Nytran, Schleicher & Schuell, Inc.), and probed with labelled moCRP cDNA. A single clone giving a positive hybridization signal was amplified, and the recombinant pBluescript plasmid was obtained by *in vivo* excision. The recombinant plasmid was designated pMCSK⁺.

Preparation of chloramphenicol acetyltransferase (CAT) constructs

The plasmid pCBPX-MCRP was constructed by cloning an *NcoI*–*ApaI* fragment from the 5' flanking region of the moCRP gene from position –2.3 kb to position –3 bp from the transcriptional start site, in front of the bacterial CAT gene in the

promoterless plasmid vector pCATBasic (Promega, Madison, WI, U.S.A.). Constructs of pCBPX-MCRP with truncated 5' regions were generated with exonuclease III/Mung-bean nuclease using the procedures provided with the Exo/Mung DNA-Sequencing System from Stratagene. The pCBPX-MCRP was first digested with *SaI*I and *Pst*I and treated with exonuclease III. The DNA fragments were then made blunt-ended and religated.

DNA sequencing

The insert in pCBPX-7, one of the truncated constructs containing ~1 kb insert of DNA, was sequenced using T3 primers and the 2.0 version of Sequenase (U.S. Biochemical Corp., Cleveland, OH, U.S.A.) by the dideoxy chain-termination method, as modified by the manufacturer for sequencing double-stranded templates [48].

Transient transfection assays

Plasmid DNA (20 μ g/100 mm plate) was transfected as a calcium-phosphate precipitate into the various tissue-culture cell lines and the cells were given a glycerol (15%, v/v) shock 4 h after adding the DNA [49]. The hepatoma cell lines were transfected on the day that they attained approx. 50% confluence. Transfected cells were harvested 40 h after transfection and suspended in 0.25 M Tris/HCl (pH 7.8) [50]. Cell lysates were prepared by three cycles of freezing and thawing and then centrifuging at 13000 g for 5 min. The supernatant fluids were collected and tested for β -galactosidase activity [47] and CAT activity [50]. The cell extracts for CAT assays were standardized using the amount of β -galactosidase activity from the same cells co-transfected with pSV β Gal (Promega, Madison, WI, U.S.A.). The amount of radioactivity in the substrate and in the products of the CAT assay was measured on a Betascope 603 blot analyser (Betagen Corporation, Waltham, MA, U.S.A.), and was used to calculate the relative CAT activity.

Nuclear extract preparation and gel-retardation assays

Nuclear extracts from liver, lung, kidney and spleen of C57BL/6N mice were prepared using the methods of Dignam et al. [51]. The gel-retardation assays were done with 15 μ g of nuclear protein extracted from each tissue, 3 μ g of poly(dI-dC) and 0.5–1 ng of the 32 P-labelled DNA fragment (~20000 c.p.m.) in the reaction mixture [52]. The DNA probe was prepared as follows: the sequence from position –226 to –45 of the moCRP promoter was inserted into pCATBasic between the *Hind*III and *Xma*I sites (pCBHX). The fragment was excised with *Pst*I and *Xma*I, and labelled at the *Xma*I site with [α - 32 P]dCTP. For competition experiments, different amounts of the oligomer and of the complementary strand for the proximal element of the human albumin promoter (H-ALB-PE) were added to the reaction [53]. The oligomer synthesized from the H-ALB-PE is 5'-GGGATGAGTCTAGTTAATAATCTACAATT-3'. The underlined sequence corresponds to the HNF-1 recognition site. The oligomers for the simian virus 40 (SV40) promoter (SP1), nuclear factor-1/CCAAT-binding transcription factor (NF1/CTF) and activator protein-1 (AP1) were purchased from Stratagene and used as competitors. The samples were separated by PAGE by the methods of Carthew et al. [52] using low-ionic-strength buffer (6.7 mM Tris/HCl, pH 7.9, 1 mM EDTA, pH 8.0, and 3.3 mM sodium acetate, pH 7.9).

Figure 1 Restriction map of the moCRP gene (a) and the nucleotide sequence of the 5' flanking region of the gene (b)

(a) The diagonally hatched blocks and empty block correspond to the two exons and the single intron, respectively. The restriction sites shown are: *EcoRI* (E), *HindIII* (H), *ApaI* (A), *NcoI* (N) and *DraI* (D). (b) The pCBPX-MCRP plasmid containing the -2.3 kb to -3 bp fragment (*NcoI* site to *ApaI* site) was used to generate a series of deletions in the 5' to 3' direction. The pCAT plasmids containing several different truncated 5' regions were then used for sequencing. The sequence represents the entire insert present in one of the CAT plasmids (pCBPX-7) containing the upstream sequence from -1083 to -3 bp from the transcriptional start site. The TATA box and GRE are underlined once; the HNF-1 sites are underlined twice. The C/EBP recognition sites (IL-1 REs) and IL-6 REs are boxed. The HSE is designated by asterisks.

RESULTS

Isolation of a mouse CRP genomic clone

Using a ³²P-labelled *PstI*-*SmaI* fragment from the mouse CRP cDNA clone MCRP-1 as a probe, a Southern blot was performed using genomic DNA isolated from the liver of a C57BL/6 mouse. The *EcoRI* digest of the mouse genomic DNA yielded a single band of ~ 5 kb. Size-selected DNA from the gel containing the 5 kb *EcoRI* fragments was isolated and used to generate a liver genomic library that was screened by hybridization with mouse CRP cDNA under high-stringency conditions. A single positive clone was obtained from the screening of 1.5×10^5 plaques. The cloned DNA fragment was digested with various restriction enzymes, blotted and probed with the cDNA for moCRP. The restriction map of the moCRP genomic DNA fragment is shown in Figure 1(a). The 2.3 kb *NcoI*-*ApaI* fragment representing most of the 5' flanking region of the gene was used to prepare a plasmid construct with the reporter CAT gene in pCATBasic; the construct was termed pCBPX-MCRP. A series of 5' deletion mutants were generated from the pCBPX-MCRP construct and used for sequencing the 5' flanking region and testing it for promoter activity.

Sequence of the 5' flanking region of the mouse CRP gene

The nucleotide sequence of the 5' flanking region of the moCRP gene is shown in Figure 1(b). The sequence was obtained from the pCBPX-7 construct that contained the upstream sequence of

the moCRP gene from -1083 to -3 bp from the transcriptional start site. This genomic DNA sequence for moCRP differed at one nucleotide from that reported by Ohnishi et al. [10] for a partial 5' flanking sequence that begins at position -226: T was observed at position -116, whereas they report C at this position. The sequence of the moCRP gene from the -3 site through the intron was completely identical to the published sequence for this region of the gene [10]. Upstream elements present in this DNA sequence that could potentially regulate expression of the moCRP gene were identified by scanning for consensus sequences of several tissue-specific elements, as well as other elements that might respond to extracellular signals generated during an acute inflammatory response.

Heat-shock elements

The first element that was looked for was the heat-shock consensus sequence or heat-shock element (HSE), since three such elements are present downstream from the transcriptional initiation site in the huCRP gene [8,9]. There is one HSE in the 5' untranslated portion of the moCRP genomic DNA that is similar to the *Drosophila* HSE: NTTCNNGAAN [54]. This HSE is located between positions -143 and -134 and has the sequence gTTCaGAAT (Figure 1b). According to the published mouse CRP cDNA sequence [11], two additional HSEs with the CTNGAANNTTCNAG motif [55] are located in the transcribed portion of the moCRP gene between nucleotides +11 and +23 (CcaGgAcTcTtG), and +27 and +38 (tTGAtctTTCAG). The

Figure 2 Cell type-specific expression of the pCBPX-MCRP construct containing positions -2.3 kb to -3 bp of the moCRP gene-promoter region

Transcriptional activity was assayed after the transfection of either the hepatoma cell line Hep 3B.2 or the epithelial cell line HeLa. The pCATControl plasmid contained the SV40 promoter and enhancer sequences, whereas pCATBasic lacked the promoter and enhancer sequences. Each plasmid construct was co-transfected with the pSV β Gal plasmid, and the CAT assays were standardized on the basis of β -galactosidase activity

HSEs in the moCRP gene did not influence transcription, since the exposure of primary mouse hepatocytes in culture to a temperature of 41°C failed to increase the mRNA signal on a Northern blot even in the presence of IL-1 or IL-6, although the mRNA coding for hsp70 was induced (results not shown).

Glucocorticoid- and cytokine-responsive elements

The 15mer consensus sequence for the glucocorticoid-response element (GRE), GGTACANNNTGTTCT, was originally identified by direct binding studies with purified hormone receptor [56]. There are two very similar GRE sequences in the 5' portion of the moCRP gene at positions -424 to -410 (GGGAAAgttGTTCT), and -153 to -139 (tGatttctcGTTCT) (Figure 1b). Sequences that are responsible for responsiveness to cytokines were also searched for in the 5' flanking region. The hexanucleotide sequence CTGGGA is present in a number of IL-6-responsive genes and is required for IL-6-induced expression of several APRs [18–20,39,43]. This sequence occurs once at positions -404 to -399 (Figure 1b). The TTGAGAAAT sequence is a C/EBP recognition site and is essential for the IL-1 responsiveness of the promoter from the human C3 gene, an APR [42]. Two similar C/EBP sequences in the promoter region of the moCRP gene are found between positions -75 and -67 (TTagGAAT), and -369 and -361 (TaTGGAAAa; Figure 1b). Nuclear factor for the IL-6 gene (NF-IL6) is a nuclear DNA-binding protein and transcription factor that recognizes an IL-1 responsive element within the IL-6 gene, and which binds to the C/EBP site [57]. The presence of these various elements indicates that moCRP gene expression in liver hepatocytes is likely to be influenced and regulated by exogenous cytokines and steroids.

HNF-1-binding elements

A potential regulatory element that is present in the 5' flanking sequence that is recognized by the liver-specific transcription factor HNF-1 is the nearly palindromic consensus sequence GTTAATNATTAAC [29]. There are two regions in the 5'

portion of the moCRP gene that are very similar to this HNF-1-recognition sequence: positions -172 to -160 (tgTAATaAaTAAC), and -72 to -60 (GgaAATcATTtAC; Figure 1b). The latter HNF-1 sequence overlaps the C/EBP, IL-1 responsive element. The presence of two C/EBP binding sites and two HNF-1 recognition sequences suggests that expression of the moCRP gene is likely to be tissue-specific, and we therefore examined this. To identify such functional *cis*-acting elements in the 5' flanking region of the moCRP gene, we used the human hepatoma cell line Hep 3B.2, because of its efficient uptake of DNA and its similarity to normal liver hepatocytes in terms of hormone responsiveness and the presence of liver-specific transcription factors [58].

Cell-specific expression of the moCRP gene

A previous study from this group only detected moCRP mRNA in the liver [17]. Since the activity of tissue-specific genes is controlled primarily at the level of the initiation of transcription, the 5' portion of the moCRP gene was examined for its ability to drive expression in the human hepatocyte cell line Hep 3B.2 and in the epithelial cell line HeLa using transient-transfection assays. A 2.3 kb fragment of the moCRP gene, extending from the *Nco*I site at -2.3 kb to the *Apa*I site at -3 bp (Figure 1a), was ligated into a promoterless plasmid containing the bacterial CAT gene and designated pCBPX-MCRP. The construct was co-transfected into each cell line with an equal quantity of the pSV β Gal plasmid, in which the transcription of the β -galactosidase gene is directed by the SV40 promoter. The pSV β Gal plasmid served as a control for the efficiency of transfection and the CAT assays were standardized on the basis of their β -galactosidase activity. The pCBPX-MCRP plasmid generated levels of CAT activity that were indicative of an enhancer-driven promoter in the hepatocyte cell line Hep 3B.2, but not in HeLa cells, indicating that the cell-specific moCRP gene enhancer and promoter elements are contained within the -2.3 kb 5' segment (Figure 2).

Figure 3 Analysis of the transcription of moCRP-CAT fusion genes in Hep 3B.2 cells and the effect of 5' deletions on the transcriptional activity of the moCRP gene promoter

(a) A series of 5' deletion mutants constructed from pCBPX-MCRP containing 2.3 kb of the 5' flanking region of the moCRP gene were assayed for CAT activity after co-transfection with the pSVβ Gal plasmid. (b) A series of constructs containing — 2.3 kb (pCBPX-MCRP) to — 32 bp (5'Δ-32) to — 3 bp (in both constructs) of the moCRP 5' flanking sequences linked to the reporter CAT gene were tested for transcriptional activity after transfection into Hep 3B.2 cells. The relative CAT activities were standardized with respect to the β-galactosidase activity in the extract. Data shown are the averages of four experiments.

Cis-acting regulatory regions in the moCRP promoter

To identify specific sequences within the 5' upstream 2.3 kb fragment of the moCRP gene that are essential for transcription, deletions were made in the 5' to 3' direction using exonuclease III/Mung-bean nuclease, and the pCAT constructs with the truncated 5' segments of the promoter region generated were then evaluated by transient transfection. The 5'Δ-220 construct containing nucleotides — 220 to — 3 expressed almost the same amount of CAT activity as the pCBPX-MCRP construct containing the entire 2.3 kb fragment, indicating that the constitutive expression requires at most 220 bp of the 5'-flanking sequence of the moCRP gene (Figures 3a and 3b). The pCAT construct 5'Δ-153 showed a 75 % decrease in transcriptional activity compared with the 5'Δ-220 construct; in addition, construct 5'Δ-50 showed an 85 % decrease in transcriptional activity compared with the construct 5'Δ-90 (Figures 3a and 3b). The data indicate that the moCRP gene-promoter region contains at least two cell-specific elements between nucleotides — 220 and — 153, and — 90 and — 50. Two shorter constructs, 5'Δ-50 and 5'Δ-32, that still contain the TATA box region, displayed extremely low transcriptional activity (Figure 3a). Since the two elements are

present in the — 220 and — 50 upstream region and overlap the two HNF-1 elements, we tested for the presence of nuclear proteins that are specific for the HNF-1 element.

Recognition of the moCRP promoter by a liver-specific nuclear protein(s)

Gel-retardation assays with nuclear extracts from several different tissues of C57BL/6N mice were done to test for the presence of tissue-specific nuclear proteins, such as HNF-1, using the DNA fragment containing nucleotides — 226 to — 45. A complex with the labelled DNA fragment was obtained with the nuclear extract from the liver, but not with that from other organs (Figure 4a). To test whether HNF-1 was involved in binding to the probe, a competition assay was done with a double-stranded H-ALB-PE oligonucleotide that contains the HNF-1 consensus sequence (see the Materials and methods section) [29,53]. The mobility shift obtained with the probe from the moCRP gene was abolished when the H-ALB-PE oligonucleotide was used as the competitor (Figure 4b). By contrast, the SP1, NF1/CTF and AP1 double-stranded oligomers, which are recognition sites for other transcription

Figure 4 Binding of hepatocyte nuclear factor(s) to the promoter region of the moCRP gene

(a) Gel-retardation assay with nuclear extracts from different mouse tissues and the labelled DNA containing nucleotides -226 to -45 of the moCRP promoter region. (b) Competition for binding of HNF-1 to the moCRP promoter by H-ALB-PE. The labelled 5' flanking segment (nucleotides -226 to -45) of the moCRP gene containing two HNF-1-binding sites was incubated with a liver nuclear extract and different amounts of the unlabelled oligonucleotide H-ALB-PE, containing the albumin gene HNF-1-binding site. (c) Competition assay with the above labelled DNA probe incubated with a liver nuclear extract and a 100-fold molar excess of double-stranded oligonucleotides of unrelated recognition elements as competitors. Abbreviations used: F, free probe; B, complex formed between the nuclear proteins and the probe.

factors, did not alter the mobility shift with the -226 to -45 gene fragment (Figure 4c). The competition observed with the H-ALB-PE indicates that the binding was specific for one or both of the HNF-1 consensus sequences that are present in the promoter region of the moCRP gene.

DISCUSSION

CRP is a cyclic pentamer that is composed of five identical subunits of 23.5 kDa and therefore is a member of a group of proteins termed pentraxins, all of which possess a characteristic flat, symmetrical discoid arrangement of their subunits [3,5]. The gene organization for CRP is conserved; however, there is considerable variation among mammals in the extent to which the blood levels of CRP increase in the inflammatory response, for example human CRP increases from 100 ng/ml to several hundred μ g/ml, whereas moCRP increases from the same baseline level to only ~ 2 μ g/ml [2,6]. Although the magnitude of the human CRP response is typical of most mammals, the significance of this increase is not known [1,2]. The extent of the increase in moCRP seems likely to be a function of the gene structure, since the human CRP gene is expressed as a major APR in transgenic mice [59]. The other pentraxin, SAP, is a major APR (100-fold increase) in the mouse, but not in other vertebrate species [12,14]. Rather than dismissing the mouse CRP response as an evolutionary oddity, we attempted to gain insight into the moCRP gene expression by cloning the gene from a genomic library to obtain a 5' flanking region of sufficient length to examine it for functional *cis*-acting elements that might contribute to the gene

expression in the context of a systemic inflammatory response, when cytokines and glucorticoids are more abundant in the portal circulation.

The 5' flanking region of the moCRP gene does contain tissue-specific elements such as HNF-1 and C/EBP binding sites, as well as HSEs, GREs and IL-6-responsive elements. Since fever accompanies host inflammatory responses, CRP gene expression might be expected to be influenced by an increase in temperature. However, we did not observe either the induction of moCRP or an increase in moCRP message levels in primary cultures of mouse hepatocytes heat-shocked at 41 °C, even with the addition of the inflammatory cytokines, IL-1 and IL-6. This agrees with the observation of others that the three HSEs in the huCRP gene do not influence transcription [60]. It has been proposed that the position of the HSEs downstream from the transcriptional start site in the huCRP gene is not consistent with a heat-shock influence on transcription [9,60]. The *cis*-acting IL-6-responsive element (IL6-RE) in the promoter region of the human genes for haptoglobin [61], haemopexin [62] and CRP [63] appears to be both necessary and sufficient for IL-6 induction of transcription. Although the moCRP gene also has the CTGGGA core sequence of the IL6-RE that was originally described for the rat α_2 -macroglobulin gene [64], neither recombinant huIL-6 nor moIL-6 triggered moCRP gene expression in isolated liver hepatocytes, although the mRNA for moSAP was substantially increased [17]. Therefore, the mouse hepatocytes contain the IL-6-dependent DNA-binding protein that defines the family of leucine-zipper proteins closely related to C/EBP [39]. One explanation for the lack of IL-6

responsiveness is that the 5' flanking region within the moCRP gene contains negative element(s) interfering with the interaction of IL-6-dependent DNA-binding protein with the IL-6 RE. Transfection studies using the same constructs described here with several mouse hepatoma cell lines did not result in a different outcome in terms of the tissue-specific expression or the IL-1 responsiveness compared with the human Hep 3B.2 cell line [17].

The C/EBP/NF-IL6 consensus sequences at positions -369 to -361, and -75 to -67, in the moCRP gene possess significant sequence similarity to the IL-1-responsive element both in the human IL-6 gene [57] and in the human C3 gene [42]. Although the moCRP gene contains this IL-1-responsive element, and the mRNA for moCRP accumulates in primary mouse hepatocytes in response to IL-1, it is unlikely that these two elements are active at the transcriptional level, since transfection experiments with the various pCAT constructs described in this paper failed to detect a significant increase in gene expression after the exposure of Hep 3B.2 cells to IL-1 [17]. Nevertheless, IL-1 might influence the level of mRNA translation and/or mRNA stability, especially since Ganter et al. [19] could show that IL-1 acts at the post-transcriptional level on the huCRP gene.

Corticosteroids such as dexamethasone have been shown to enhance the cytokine-driven induction of a subset of APR genes: C1-inhibitor, serum amyloid A, CRP and SAP in the PLC/PRF/5 human hepatoma cell line [65], and CRP and serum amyloid A in Hep 3B.2 cells [66]. Recent findings by Hocke et al. [67] demonstrated that the synergistic action of glucocorticoids and IL-6 is mediated through the IL-6 RE of the rat α_2 -macroglobulin gene. However, many different concentrations of glucocorticoids in association with IL-6 failed to induce gene expression using Hep 3B.2 cells transfected with the moCRP gene-promoter region (2.3 kb) constructs (N.-O. Ku and R.F. Mortensen, unpublished work).

Previously we reported that the mRNA for moCRP was present at significant constitutive levels in mouse hepatocytes, but not in other cells or tissues [17], and was clearly inducible in mice [11]. In the present study, we identify DNA sequences within the 5' region of the moCRP gene that serve as binding sites for HNF-1, a transcription factor that is found only in fully differentiated hepatocytes [29]. Since the sequences between nucleotides -220 and -153 and between nucleotides -90 and -50 that confer tissue-specific expression overlap with a single C/EBP, IL-1-responsive element in the second sequence, we cannot rule out a contribution of this element to the moCRP gene expression. A comparison of the moCRP promoter region with that of other hepatocyte genes reveals significant sequence similarity with the HNF-1 sites in the genes for human albumin [53], human α_1 -antitrypsin [36], rat β -fibrinogen [36] and human CRP [37] (Figure 5). Moreover, we found that the relevant portion of the 5' region of the moCRP gene can form a stable complex with nuclear-protein extracts from liver, but not from lung, kidney or spleen; furthermore, complex formation was specifically inhibited by the addition of a double-stranded oligonucleotide that contained a copy of the HNF-1-binding site. Therefore, the 5' region of the moCRP gene contains functional HNF-1-binding sites.

Ultimately, insight into the molecular basis of the acute-phase CRP response will be needed to determine if CRP possesses a unique function, or simply functions in a manner analogous to specific antibody (that is, in complement activation and opsonization) in the pre-immune phase of a host response. Since the genes for both CRP and SAP [68,69] in the mouse have been cloned and mapped to chromosome 1, the mouse might serve as a model for examining the co-ordinated regulation of

Figure 5 Comparison of the HNF-1-binding consensus sequences within the promoter of several APR genes with the two HNF-1 sequences present in the moCRP gene promoter region

The asterisks designate the nucleotides that differ from the HNF-1 consensus. The consensus sequence is denoted by an arrow.

expression of these two genes, which are present in the same syntenic region [7]. A direct comparison of the sequences of the 5' flanking segments of the moCRP with the huCRP gene by Ohnishi et al. [10] indicated an overall conservation of 69% up to nucleotide -226 from the cap site of the mouse gene, as well as a highly conserved arrangement of the HSE and HNF-1 recognition sites. Therefore, the basis for the marked species differences in the acute-phase and cytokine responsiveness of the CRP gene may be revealed by chimaeric constructs using fragments that span the entire 5' flanking region through the single intron.

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