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Identification of sequences responsible for acute-phase induction of human C-reactive protein

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ABSTRACT

Human C-Reactive protein (CRP) is inducible in liver cells during acute inflammation. Around 90 bp from the 5' flanking region of the human CRP gene contain, as shown here, information to induce the expression of a linked bacterial CAT gene specifically in human hepatoma (Hep3B) cells. The promoter is induced rapidly, faithfully and at high efficiency when transfected cells are exposed to conditioned medium from lipopolysaccharide stimulated peripheral monocytes. The sequences required for inducibility are located immediately upstream to the TATA element. A DNA segment from base -121 to -50 is capable of inducing transcription from the heterologous SV40 early promoter. Induction of CRP expression is probably exerted via the binding of at least one positive trans-acting factor.

INTRODUCTION

Human C-Reactive protein (CRP) is a plasmaprotein synthesized in the liver and secreted into the plasma as a pentamer of identical subunits each with a molecular weight of 22,500 daltons, arranged in an annular disc configuration (for a review, see 1). The protein belongs therefore to the family of the pentraxins together with the serum-amyloid protein (SAP) and with the female hamster protein. The most interesting feature of CRP biosynthesis is its participation in the acute-phase reaction: human CRP plasmatic levels rise up to 1,000 fold during the course of inflammation . For this reason, monitoring of the CRP plasma levels has been extensively used in clinical practice as a means to follow the evolution of several diseases like rheumatic disease or coronaries (1).

The mechanism responsible for the regulation of CRP biosynthesis is still unresolved. CRP mRNA levels increase drammatically in liver cells during acute inflammation (2). However, it has not been possible to show whether regulation of gene expression takes place mainly at the transcriptional level or posttranscriptionally via stabilization of the mRNA. Some results relevant to this issue have recently come from work with transgenic mice. The human CRP gene introduced into the mouse germ line behaves as a major positive acute phase reactant and nuclear run-on experiments have indicated that the rate of gene transcription is greatly enhanced during inflammation (3).

The acute phase response is a complex metabolic event which is characterized by a substantial change in the pattern of plasmaprotein production in liver cells (4). This change is mediated by a series of molecules released from cells physically associated with the focus of

inflammation, namely fibroblasts, macrophages and granulocytes. The monokines IL-1 and TNF are certainly responsible for the activation of a subset of acute phase genes (5-8). Recently, however, a new monokine called HSF has been identified which seems to be responsible for most of the changes occurring in liver during inflammation (9); this molecule is the one and the same as the previously well characterized B-cell differentiation factor BSF-2/IL-6 or interferon β_2 (IFN-β_2) (10-12).

Darlington et al.(7) have recently shown that the conditioned medium from bacterial lipopolysaccharide treated peripheral monocytes contains all the monokines responsible for hepatocyte stimulation and is therefore capable of eliciting in the human hepatoma cell line Hep3B a change in the pattern of plasmaprotein production similar to that observed *in vivo* in the liver during inflammation. CRP mRNA levels rise upon such stimulation of Hep3B cells, even though the absolute amount of CRP mRNA synthesized is lower than that observed in the living organism. More recently, Oliviero et al. (13) demonstrated that only 183 bp of the 5` flanking region of the human haptoglobin gene contain sufficient information to induce expression of a linked reporter gene when transfected into stimulated Hep3B cells.

We have used the Hep3B stimulation system in order to analyse the function of the 5` flanking region of the human CRP gene. The promoter of the CRP gene contains information sufficient to induce the expression of a linked reporter gene when transfected into human hepatoma cells. The timing and the extent of gene activation given by the CRP promoter mirrors the pattern of gene activation *in vivo*. Cotransfection competition studies identified a transacting factor governing CRP expression.

MATERIALS AND METHODS.

Cell cultures, DNA transfections and CAT assays.

Human hepatoma cell lines HepG2 and Hep3B (14) and HeLa cells were cultured as monolayers in Dulbecco`s modified Eagle`s medium supplemented with 10% fetal calf serum. Inductions were performed incubating the cells in the presence of 10% conditioned medium from LPS stimulated peripheral blood monocytes as described (7). DNA transfections were performed with the calcium phosphate precipitation tecnique (15). Transfections were done in duplicate and only one set of plates was induced for the time indicated in each experiment. CAT assays were performed as described (16). In the cotransfection competition experiment a total constant concentration of 10 μg/5 x10^5 cells/plate was used. Test plasmid was always in the quantity of 0.2 pmoles/plate. Competitor plasmid varied in the quantity of 0.2, 1.4, 2.9 and 4.4 pmoles/plate.

Plasmid construction.

Plasmid CRP-CAT1 was constructed by cloning a genomic BglII-BglII segment of the 5` flanking region of the human CRP gene from position -2,500 to position +12 with respect to the cap site, in front of the bacterial CAT gene in plasmid vector p8CAT (17). 5`Δ-786 is a derivative of CRP-CAT1 obtained removing the region upstream of position -786 by making use of a natural Xba I site. All the other 5`deletions were obtained with progressive Bal31 resection of 5`Δ-786.

3` deletions were constructed by first subcloning the 5`flanking region of the CRP gene from nucleotide -786 (XbaI) to +12 (BglII) in plasmid pEMBL130 (18) and then subjecting it to progressive resection with Bal31. The resulting clones were initially sequenced to precisely map the deletion end point and a subset of them was cloned in the Sma site of plasmid pUC19CAT2 (19).

Plasmid pEMBL-CRP -121/-50 was constructed isolating from 5`Δ-121 a DNA segment from the deletion end point to a natural HhaI site at position -50 and cloning it into pEMBL131. The same segment was also cloned in the correct orientation upstream from the SV40 early promoter in plasmid pUC19 CAT2 to give the construct -121/-50.

S1 mapping

S1 mapping was performed according to Berk and Sharp (20) as modified by Ciliberto et al. (21). All probes were obtained by in vitro elongation on M13 ss templates. For transcripts from the CRP promoter-CAT fusions a segment from 5`Δ-219 , carrying 219 bp of CRP flanking linked to the first 250 bp of theCAT gene was cloned into M13 TG131. This probe should give rise to a protected band of 282 nucleotides if the correct CRP cap site is used (22). For transcripts from the 3` deletion mutants and from the SV40 β-globin plasmid the clones have already been described (13). S1 analysis with the SV40 β-globin probe gives rise to a protected band 201 to 204 nucleotides long, and S1 analysis of the 3` deletion clones in plasmid pUC19CAT2 give rise to a protected band 309 nucleotides long (13).

RESULTS.

The 5` flanking region of the C-Reactive protein gene contains sufficient information for acute phase inducibility in human hepatoma cells.

Previous work with the human CRP gene in transgenic mice indicated that regulation of gene expression takes place mainly at the transcriptional level (3). In order to identify and characterize the DNA region responsible for this activation we used DNA mediated gene transfer in cultured cell lines. The human hepatoma cell line Hep3B, cultured for 48h in the presence of 10% conditioned medium from LPS activated peripheral monocytes, shows increased steady state mRNA levels for several plasmaproteins including CRP (7). As an initial approach we therefore isolated 2.5 kb of CRP flanking sequence and fused it to the bacterial gene for chloro-amphenicolacetyltransferase (CAT). The resulting construct, CRP-CAT 1, (fig.1) was transfected into three different cell lines: HeLa, Hep3B and HepG2, the first one of non-hepatic origin the other two of hepatic origin . Transfections were done in duplicate but only one set of plates was treated with 10% conditioned medium from monocytes for the following 48 h. The results (fig.2) show that CRP/CAT 1 is not expressed in any of the cell lines in the absence of stimulation, but is inducible to a various extent only in the two hepatoma cell lines; Hep3B appears to be at least 20-fold more efficient than HepG2.

From this result we conclude that the 2,500 bases of CRP 5` flanking region contain information for induction of transcription *in vitro* similar to the acute phase response *in vivo*.

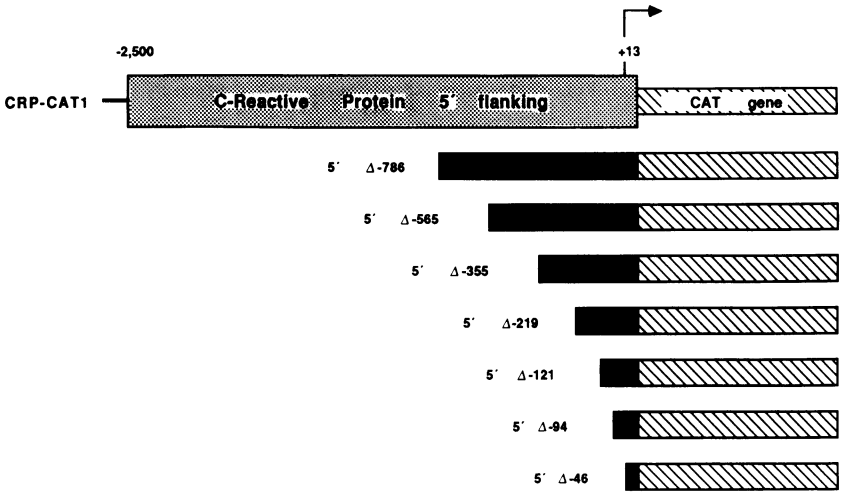

Fig.1. Schematic representation of CRP-CAT fusions. The original 2.5 kb DNA segment carrying the 5'flanking region of the CRP gene in plasmid CRP-CAT 1 is represented with a solid grey bar. Black bars represent the remaining portion of the 5'flanking in the respective 5'deletions. Hatched bars represent the CAT coding region. The arrow indicates the direction of transcription.

Although the induction is confined to hepatic cells we do not know if the cell specificity is dependent on a different distribution of intra-cellular transacting factor(s) responsible for gene activation or if cells other than hepatocytes lack the appropriate cell surface receptors for hepatocyte stimulating factors secreted by monocytes. The transfected promoter seems to be much more sensitive to induction than the endogenous gene; in fact the induced CRP-CAT1 construct is expressed upon induction as efficiently as hepatocyte-specific α_1-antitrypsin (α_1-AT)-CAT fusions (21,19 and our data not shown), whereas the endogenous CRP mRNA is far less abundant than the endogenous α_1-AT mRNA (7 and our observations, not shown).

The transfected CRP promoter is rapidly induced

Ciliberto et al.(3) showed recently that the human C-reactive protein gene introduced into the mouse genetic context is transcribed efficiently only during the course of inflammation. The timing of gene activation is very rapid: CRP mRNA in liver cells is already detectable 2 hours after i.p. injection of bacterial LPS; the maximal RNA values are reached after 9 hours but RNA disappears quickly and is almost undetectable after 24 h. In contrast, induction of CRP synthesis in hepatoma cells (7) only reveals CRP mRNA after 24 to 36 h of continuous stimulation with monocyte conditioned medium. Since the expression of the endogenous CRP gene is poor compared with that driven by a transfected promoter, an assay based on transfected genes may be a sensitive way of studying the mechanisms of gene activation during acute inflammation. We have therefore transfected CRP-CAT1 and induced cells for different times with monocyte conditioned medium.

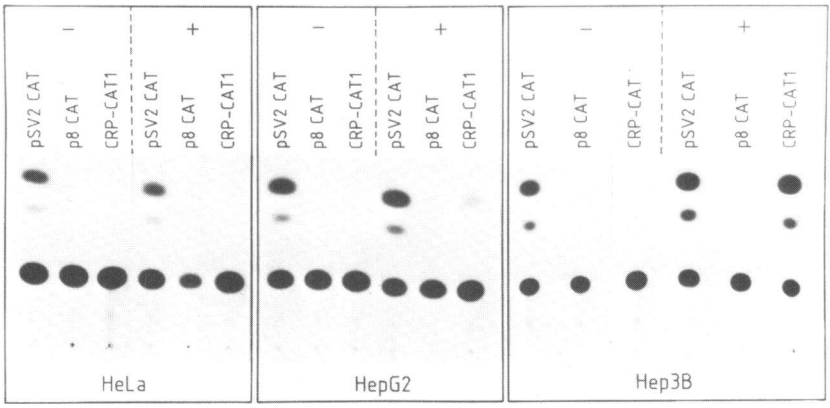

Fig.2. Expression of CRP-CAT 1 in variuos cell lines. pSVCAT2 carries the SV40 enhancer (16). p8CAT carries the CAT coding region without any enhancer and promoter. - indicates absence of conditioned medium, + presence of 10% conditioned medium from LPS stimulated peripheral monocytes.

RNA was extracted and subjected to quantitative S_1 analysis. The results are shown in fig.3. As expected, transcription is not detectable from the CRP promoter in unstimulated cells but abundant quantities of transcripts starting from the CRP cap site are produced already 2h after of exposure to monocyte medium. RNA concentration reaches a maximum at 24 h and starts to decline at 36 h. It is not possible to directly compare the conditions of stimulation in the whole animal with those in cell cultures because the monokines responsible for CRP expression might have a completely different half-life *in vivo* and *in vitro*. However, in both cases gene induction occurs very rapidly thus indicating that the Hep3B system with the transfected gene is faithfully reproducing the conditions of hepatocyte stimulation observed in the living animal during inflammation and that the CRP 5` flanking region in the CAT construct behaves *in vitro* like the intact gene *in vivo*.

Definition of the minimal sequence required for CRP induction

To better define the sequences responsible for the inducibility we generated a series of progressive 5` deletions (fig.1). These constructs were transfected into Hep3B cells and CAT activity was assayed after 48 h with or without stimulation with monocyte supernatant. The results are shown in fig.4 A . Constant inducibility is mantained down to deletion 5'Δ-94. The deletion mutant 5`Δ-46 does not show a significant response to the conditioned medium and its activity after induction is comparable to that of the control plasmid p8CAT, in which the CAT gene is not linked to any pol II promoter. CAT activity is not induced in the control plasmid pSV2CAT in which the reference gene is coupled to SV40 early promoter and enhancer.The absolute amount of CAT conversion found in pSV2CAT in the experiment shown in fig. 2 is higher than that in fig. 3 because in the former we have tranfected 10μg of active DNA/5x10^5 cells,whereas in the latter only 1 μg of active DNA plus 9 μg of plasmid vector pEMBL8+ as carrier DNA. In order to precisely quantitate

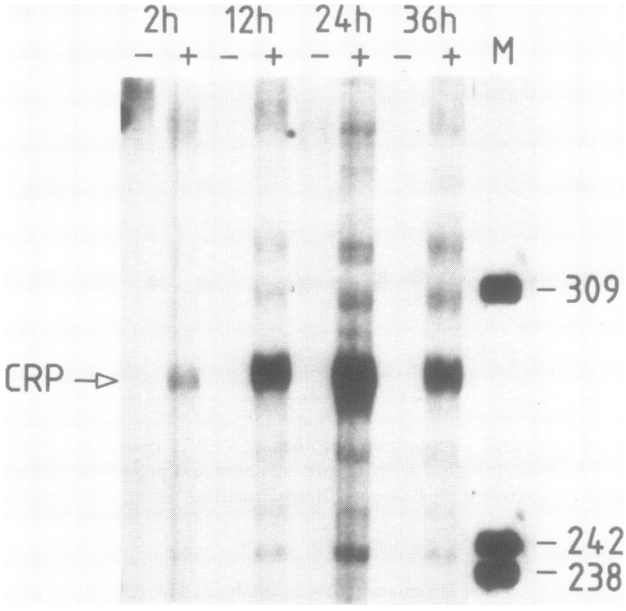

Fig.3. Time course of CRP-CAT 1 expression. CRP-CAT 1 was transfected into Hep3B and the cells were unstimulated or stimulated with monocyte supernatant (- or + respectively). RNA was extracted and subjected to S1 analysis (see materials and methods). After S1 digestion the protected DNA fragments were separated on 6% TBE-7M urea polyacrylamide gel. M is DNA size marker pBR322 digested with HpaII.

the level of inducibility of the CRP promoter we analysed the RNA transcribed from the last three deletions by quantitative S_1 analysis. In this last case we cotransfected the CRP-CAT fusions with an SV40-βglobin construct as an internal control for the efficiency of transfection. This construct is efficiently expressed in Hep3B cells and its transcription is not significantly influenced by the monocyte supernatant (13). The results of the S_1 analysis (fig.4B) confirm the CAT expression data.

Deletions 5`Δ-121 and 5`Δ-94 are only efficiently transcribed after stimulation. Transcription starts at the level of the true CRP cap-site and no faithful transcription is observed in any of the deletions in the absence of stimulation. The sensitivity of our S_1 assay would have detected a signal as low as 1/100 of that observed in the stimulated cells. Therefore we believe that the CRP promoter is silent under normal conditions and that 94 bp of 5` flanking sequence contain enough information to confer full stimulation of gene expression in the hepatoma system. Furthermore, the low level of CAT activity that we observe with some of our constructs is probably attributable to spurious transcripts starting in the vector sequence (see the asterisk in fig. 4B). The high levels of spurious upstream transcripts that we observe in the absence of stimulus in constructs 5' Δ-121 and 5' Δ-94 are strongly reduced after addition of monocyte supernatant as if the activation of the CRP promoter is able to polarize plasmid transcription only from the true cap-site.

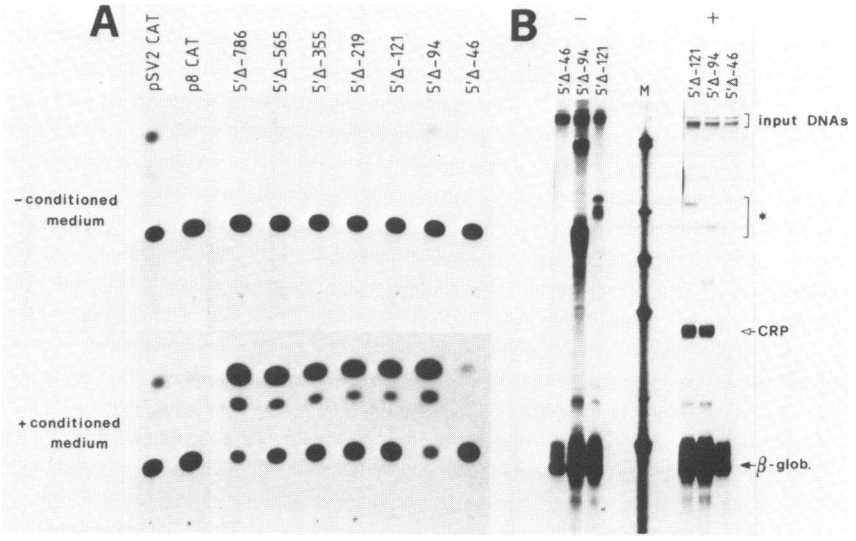

Fig.4. Expression of 5`deletions mutants. A) CAT assay on the various CRP-CAT 5` deletion mutants; B) S1 analysis of deletions 5` Δ-121, 5` Δ-94 and 5` Δ-46. M is DNA size marker pBR322 digested with HinfI. The asterisk (*) indicates spurious transcripts starting in the vector sequences upstream from the true CRP cap site.

The CRP 5` flanking region is able to confer inducibility to a heterologous promoter.

The 5` flanking region of the CRP gene from nucleotide -786 to nucleotide +16 was subjected to progressive resection from the 3` side and the resulting deletions were cloned upstream from the early promoter of SV40 in plasmid pUC19CAT2 (19)(fig. 5A). This plasmid carries the SV40 early promoter from the Sph I site (pos.133) to the HindIII site (pos.5172) (23) in front of the bacterial CAT gene. Transfection of control pUC19CAT2 plasmid gives rise to a low level of CAT activity. In addition, induction of Hep3B cells transfected with this plasmid leads only to a minor (2 to 3-fold) increase of CAT expression (13). We therefore transfected our CRP/SV40 CAT fusions into unstimulated and stimulated Hep3B cells to test if the inducibility can be transferred to a heterologous promoter and to further narrow down the DNA region responsible for this effect. The results are shown in fig. 5B and C. The first construct of the series (3`Δ-41) , in which the TATA element and the sequences downstream are removed is able to confer inducibility to the SV40 promoter. This is decreased in the following deletions and completely abolished when the 137 bp upstream to the cap-site are deleted. Furthermore, the DNA segment from nucleotide -121 to nucleotide -50 is capable of giving full induction of CAT expression in this system. We have determined the activity of 3`Δ-138 and -121/-50 by $S_1$ analysis (fig 5D). 3`Δ-138 does not give rise to any detectable transcript either with or without stimulation, whereas -121/-50 is capable of inducing the production of an RNA of the expected size (see materials and methods) only after treatment of the cells with monocyte supernatant (fig 5D). We therefore conclude that most of the

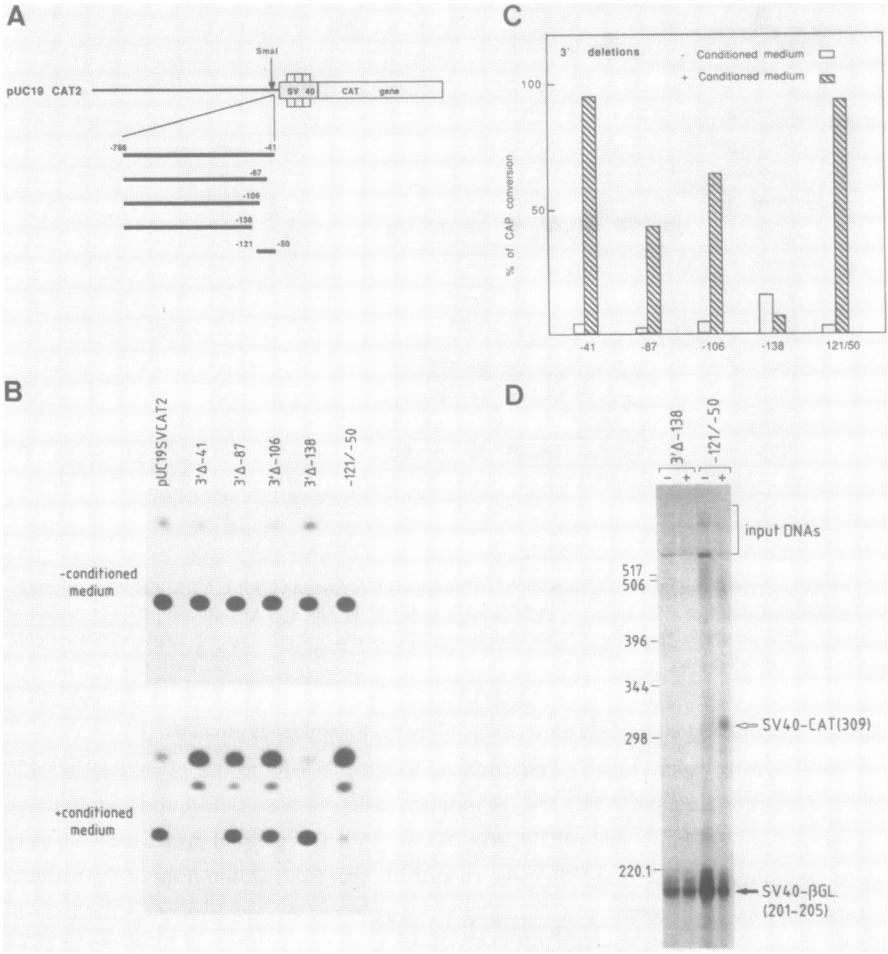

Fig.5. Expression of 3`deletion mutants. A) Schematic representation of CRP 3`deletion mutants. Plasmid pUC19CAT2 has been described (19). Solid black bars represent the portion of CRP 5`flanking region contained in each construct; B) CAT assay on the 3`deletion mutants; C) Tabulation of the result of the CAT assay chromatography. The spots shown in panel B were cut and counted and the results are represented as % of CAP conversion; D) S1 analysis of constricts 3`Δ-138 and -121/-50

information for induction in hepatoma cells must be contained downstream of nucleotide -137. However, some redundancy of signals is probably present in this region because while the deletion 5' Δ-94 is fully inducible, the deletion from the 3` side of the first 87 or 106 bp reduce but do not completely abolish inducibility.

A positive control mechanism is responsible for CRP gene activation.

The increased transcription of the CRP promoter during the acute phase response might be

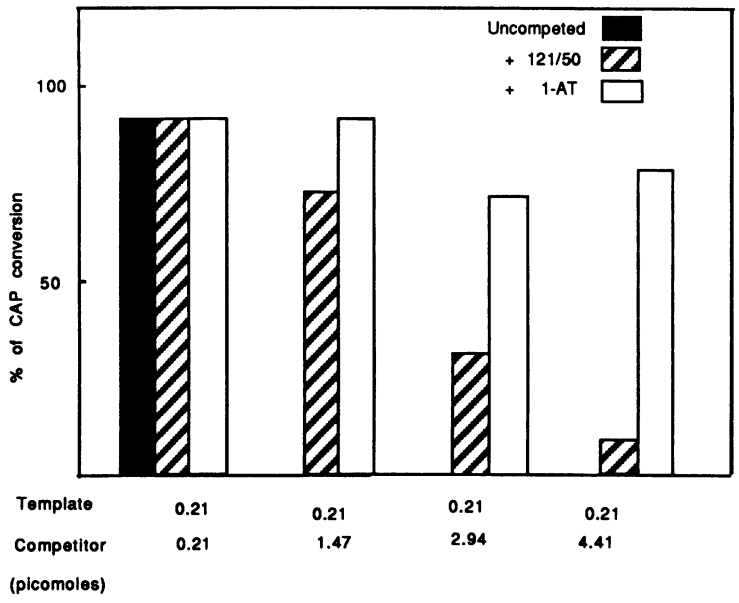

Fig.6 Cotransfection-competition Assay. As test DNA we used 5`deletion mutant 5`Δ-121 at the concentration of 0.2 pmol/5 x 10^5 cells. As competitors we used plasmids pEMBL -121/-50 or α_1 -488 (19). The results of the CAT assay are reported as percent of CAP conversion.

due either to the loss of a negative control or to the induction of one or more positive trans acting factor(s). The existence of a positive trans-acting factor can be demonstrated in competition experiments such as those performed by Schöler and Grüss (21). The deletion 5'Δ-121 was cotransfected together with two different plasmids at increasing concentrations with respect to the test plasmid (1-fold, 7-fold, 14-fold and 21-fold molar excess of competitor over the test plasmid). The results are shown in fig.6 . Only plasmid pEMBL-CRP 121/50 carrying a small segment of the CRP promoter from nucleotide -121 to nucleotide -50 is capable of competing the activity of the test plasmid in conditions of cell stimulation. The 5`flanking region of the α_1-AT gene (plasmid α_1/-488) which has been shown to be actively transcribed in human hepatoma cells (19) was, however, inactive as competitor. The same competition experiment ,when performed in the absence of monocyte supernatant, did not give rise to any activation of CRP-CAT expression (not shown).

DISCUSSION.

The human CRP gene introduced into the mouse germ line is highly induced during the course of experimental inflammation and nuclear run on experiments with isolated liver nuclei have indicated that the rate of transcription of this gene is greatly enhanced already a few hours after injection of a strong irritant like bacterial LPS (3). The data shown in this paper convincingly prove

that the promoter of the CRP gene contains acute phase responsive sequences which, in combination with a downstream TATA element are capable of activating transcription when the cells are stimulated with the appropriate complement of monokines.

The induction of the transfected CRP promoter occurs rapidly. In this respect it is similar to that observed for other inducible genes like the heat shock genes, c-fos, metallothionein, etc., where gene activation is dependent on the modification of a preexisting molecule leading to a change in its DNA binding affinity (25-27). Although we have not yet studied the requirements for ongoing protein synthesis for CRP induction a similar mechanism might be the basis of the activation of CRP gene expression during inflammation.

The transfected gene is much more sensitive to induction than the endogenous gene in Hep3B cells and its degree and timing of activation is comparable to that observed in the living organism (3). The relative inactivity of the endogenous CRP gene in Hep3B might be the consequence of methylation or changes in the structure of the chromatin which renders it partially inaccessible to the activating factor(s). In fact, we have never been able to detect DNase I hypersensitive sites in the promoter region of the endogenous CRP gene in these cells. The availability of a very sensitive assay for transfected CRP gene expression allows on the other hand a detailed study of the involvement of individual monokines in the activation of its expression.

Independent work on other acute phase response genes has shown both for the haptoglobin gene (13) and for the complement factor B (R.Arcone, D.Campbell and G.Ciliberto, unpublished), that approximately 200 bp upstream from the cap site contain sufficient information for acute phase inducibility in hepatoma cells. A computer search revealed the presence of short stretches of similarity between the promoter region of these three genes (Haptoglobin, factor B and CRP). This finding is even more intriguing when combined with the recent observation that these three genes are all induced in hepatoma cells by recombinant interleukin-6 (28). At present we do not yet know if these blocks of similarity include nucleotides important for the promotion of transcription of the genes. Recent binding studies with several DNA binding proteins and a variety of promoter elements have on the other hand demonstrated that the same trans-acting factor is sometimes able to recognize DNA sequences which, apparently, do not show any similarity with each other (29). In addition, it cannot be excluded that distinct transcription factors interact directly with these promoters but are *per se* not capable of promoting transcription; they might share, however the common feature of being activated upon interaction with an external factor, common for all of them, which is not a direct DNA binding protein. The inducibility of the CRP promoter is probably dependent on one or more positive transacting factor(s) whose existence can be demonstrated by cotransfection-competition experiments. The promoter of the α_1-AT gene, a liver specific gene, whose transcription is not changed during inflammation is obviously not able to compete for the same factor(s). Competition experiments with different acute phase promoters and in particular with those, like haptoglobin and Complement Factor B, whose induction seems to depend on the activation of a common pathway may identify common trans-acting factors.

Our results obtained with the 5` and the 3` deletions allow us to define a minimal acute phase

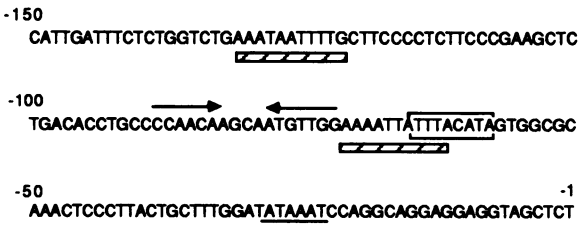

Fig.7. Sequence of the CRP promoter. The sequence of the CRP promoter was previously reported by Lei et al. (22). The TATA element is underlined; the octamer-like element is boxed; the palindrome between nucleotides -77 and -88 is indicated by convergent arrows and the two 10 bp blocks of homology are indicated by bars.

responsive element (APRE1) from nucleotide -94 to -50. A second element (APRE2) must be located upstream from this region with its 3` boundary between positions -106 and -137. This opens the possibility that protein-protein interactions are involved in CRP gene activation. The scheme with which the CRP promoter is built is therefore not very much dissimilar from that of other inducible promoters like the Drosophila heat shock gene promoters (30). Four heat shock factor binding elements plus a TATA box are required for heat shock inducibility in the whole animal but the two most proximal ones (from nucleotide -100 to -50) are sufficient to give full expression in transient transfections. The same structure is also observed in some glucocorticoid inducible promoters like the MMTV promoter where various glucocortoid-response elements (GREs) are located a few bp upstream from the TATA element (31-32). In the metallothionein MTIIA promoter a redundancy of metal responsive elements (MRE) has also been observed (33). It seems therefore a general characteristic of most of the inducible promoters that they harbour responsive elements closely condensed in a restricted area immediately upstream of the start of transcription.

In the CRP promoter the sequence between positions -65 and -58 shares some homology with the binding site for the well characterized transcription factor named Octamer Binding Factor (OBF) (fig.7) (34). The sequence in the CRP gene diverges from the canonical octamer binding site at one position (ATGTAAAT instead of ATGCAAAT). Since site-directed mutagenesis experiments aimed either at reconstituting the perfect consensus or at substituting it with a different sequence did not influence gene inducibility, a factor similar to the OBF is probably not involved (R.Arcone and G.Ciliberto, unpublished). Therefore, the relevant sequences must be located upstream and/or downstream of the octamer-like sequence. Other relevant features of the CRP promoter are the presence of a palindromic sequence between nucleotides -88 and -72 and of two blocks of homology with opposite orientation between nucleotides -135 to -126 and -71 to -62 (fig.7). At the present stage we cannot identify them with the two APREs. A precise assessement of their role in gene inducibility will be possible only after site-directed mutagenesis of the entire region.

Beside the fact that it is an inducible gene, CRP , like the other acute phase reactants, is specifically expressed in liver. The CRP-CAT fusions are specifically induced in hepatoma cells.

We have sometimes observed low levels of CAT activity for some of our 5`deletion mutants when transfected in stimulated HeLa cells but expression in these cells is always orders of magnitude lower than in Hep3B. Tissue-specific induction of the CRP promoter remains therefore unclear and several mechanisms can be postulated to account for it. One possibility is the presence of a high number of receptors for interleukin-6 specifically in hepatocytes. This cannot, however, be the only explanation since this interleukin is also capable of acting on other cells like B-lymphocytes or plasmacells which do not synthesize CRP. Alternatively, the direct DNA binding factors might be more abundantly or exclusively produced in hepatocytes. (10-12). The identification of the IL-6 receptor on one hand and the purification of trans-acting factors interacting with the CRP promoter will allow a deeper understanding of the phenomenon of the acute phase response.

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Abbreviations:

CRP	C-Reactive protein
α_1 AT	α_1-Antitripsin
i.p.	intraperitoneal
LPS	bacterial lipopolysaccharide
CAT	chloramphenicolacetyltransfrerase
OBF	octamer binding factor

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