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Synergistic *trans*-activation of the human C-reactive protein promoter by transcription factor HNF-1 binding at two distinct sites

Carlo Toniatti[1,5], Anna Demartis[1,4], Paolo Monaci[2,5], Alfredo Nicosia[2,5] and Gennaro Ciliberto[1,3,5]

[1]Dipartmento di Biochimica e Biotecnologie Mediche, II Facoltá di Medicina e Chirurgia, Via S. Pansini 5, 80131 Napoli, Italy, [2]Meyerhofstrasse 1, 6900 Heidelberg, FRG and [3]CEINGE, Centro di Ingegneria genetica, Via S. Pansini 5, 80131 Napoli, Italy.
[4]On leave of absence from Dipartimento di Fisiologia e Biochimica, Via Carducci 13, Ghezzano, Pisa, Italy
[5]Present address: IRBM Istituto di Richerche di Biologia Molecolare, Via Pontina Km. 30,600 00040 Pomezia, Roma, Italy

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The promoter region of the human C-reactive protein (CRP) gene comprises two distinct regions (APREs, for Acute Phase Responsive Elements) each one containing information necessary and sufficient for liver specific and IL-6 inducible expression in human hepatoma Hep3B cells. In this paper we show that both APREs contain a low affinity binding site for the liver specific transcription factor HNF-1/LF-B1. The two sites are separated by ~80 bp. Mutations in either of the two sites abolish inducible expression. The same effect is specifically obtained in cotransfection competition experiments when the human albumin HNF-1 site is used as competitor. However, HNF-1 is not the intranuclear mediator of IL-6 because synthetic promoters formed by multimerized copies of different HNF-1 binding sites are not transcriptionally activated by this cytokine. An expression vector encoding full length HNF-1 is capable of *trans*-activating transcription from the wild-type CRP promoter but not from mutants which have lost the ability to bind HNF-1. Moreover, the level of *trans*-activation observed with the natural promoter containing both HNF-1 binding sites is far greater than the level of mutated variants containing only one of the two sites. This result strongly suggests that two HNF-1 molecules bound simultaneously to sites distant from each other can act synergistically to activate gene expression.

Key words: C-reactive protein/liver specific gene expression/transcription factor HNF-1/*trans*-activation

Introduction

The detailed analysis of the mechanisms responsible for the transcriptional activation of RNA polymerase II dependent genes has led to the dissection of enhancer and promoter regions into multiple and discrete modules, each one able to interact with specific transcriptional activators (Jones *et al.*, 1988; Ptashne, 1988; Dynan, 1989). The binding of these *trans*-acting factors to their target sequences triggers through mechanisms still unknown the assembly and the activation of a preinitiation complex including RNA polymerase II, the TATA box binding protein TFIID and other general transcription factors which apparently do not recognize specific sequences on DNA (Wasylyk, 1988; Buratowski *et al.*, 1989; Saltzman and Weinmann, 1989). Tissue specific gene expression relies mainly on the presence of cell-type specific *trans*-acting factors, and the fine tuning of the transcriptional response is in general achieved by the coordinated interaction of sequence specific DNA binding proteins with promoter and enhancer regions (Dynan, 1989; Levine and Manley, 1989; Mitchell and Tjian, 1989).

There are several examples of genes whose transcriptional rate is influenced by stimuli external to the cell (Mitchell and Tjian, 1989). A large interest has been focused on the structure of the promoter region of inducible genes (Maniatis *et al.*, 1987). In these cases it has been possible to distinguish between modules which interact with constitutive transcription factors, and modules that bind inducible *trans*-activators (Sen and Baltimore, 1986; Angel *et al.*, 1987; Montminy and Bilezikjian, 1987; Zimarino and Wu, 1987; Fan and Maniatis, 1989; Visvanathan and Goodbourn 1989; Majello *et al.*, 1990). In these cases, therefore, full transcriptional activation results from the combined action of constitutive and inducible factors.

In hepatocytes, a large set of genes encoding plasma proteins is transcriptionally modulated during inflammation in response to various circulating cytokines, mainly interleukin-1 (IL-1) and interleukin-6 (IL-6) (reviewed by Ciliberto, 1989). In the last years our laboratory has studied the transcriptional regulation of the human CRP gene (Ciliberto *et al.*, 1987; Arcone *et al.*, 1988; Ganter *et al.*, 1989; Majello *et al.*, 1990; Toniatti *et al.*, 1990). Expression of this gene is under double hormonal control during inflammation: transcriptional by IL-6 and translational by IL-1 (Ganter *et al.*, 1989). When transfected into human hepatoma Hep3B cells the CRP promoter is silent, but can be strongly activated by recombinant IL-6 (Ganter *et al.*, 1989).

Two regions, which were named APRE1 and 2, have been shown to be important for gene expression (Arcone *et al.*, 1988). These two regions can drive inducible transcription independently from each other, even when linked to a heterologous promoter (Arcone *et al.*, 1988; Toniatti *et al.*, 1990). APRE 2 has been mapped between coordinates −90 and −46, but the precise borders of APRE1 have not been determined. With a combination of 5′ and 3′ deletions APRE1 has been mapped downstream of position −219 and upstream of position −106, but a 3′ deletion to −137 completely abolishes its activity.

A more detailed analysis of APRE2 has shown that two putative transcription factors interact with adjacent sites within this region (Majello *et al.*, 1990). The first protein, H-APF-1, which interacts with the CRP site β, is constitutively present in hepatic cells. The other, H-APF-2, binds site α and is induced by IL-6. H-APF-2, also called IL6-DBP (Poli and Cortese, 1989), has the ability to interact with the promoter region of several acute phase IL-6 inducible genes (Poli and Cortese, 1989; Majello *et al.*,

1990). Mutations in either site, α or β, strongly impair gene expression, thus indicating that both proteins play a crucial role.

The presence of a constitutive factor in hepatoma cells capable of binding to the CRP promoter prompted us to attempt a characterization of such a protein. During the last years several laboratories have identified a restricted set of hepatocyte specific or enriched transcription factors (De Simone *et al.*, 1987; Cereghini *et al.*, 1988; Courtois *et al.*, 1988; Grayson *et al.*, 1988; Hardon *et al.*, 1988; Kugler *et al.*, 1988; Landschultz *et al.*, 1988; Frain *et al.*, 1989; Baumhueter *et al.*, 1990; Mueller *et al.*, 1990). One of the best characterized is HNF-1/LF-B1/APF (thereafter referred to as HNF1). This factor interacts with the promoter region of several genes transcribed preferentially or exclusively in hepatic cells (Courtois *et al.*, 1988; Kugler *et al.*, 1988; Monaci *et al.*, 1988; Feuerman *et al.*, 1989; Frain *et al.*, 1989; Tronche *et al.*, 1989). A cDNA clone spanning the entire open reading frame of HNF-1 has been recently isolated (Frain *et al.*, 1989). It encodes a 628 amino acid protein which binds DNA as a dimer and contains a highly diverged homeodomain (Nicosia *et al.*, 1990).

Binding of HNF-1 is necessary in natural promoters and sufficient in synthetic promoters to drive the expression of liver specific genes both in *in vivo* and *in vitro* systems (De Simone *et al.*, 1987; Heard *et al.*, 1987; Monaci *et al.*, 1988; Schorrp *et al.*, 1988; Feureman *et al.*, 1989; Lichtsteiner and Schibler, 1989; Maire *et al.*, 1989). In addition, purified or recombinant HNF1 protein enhances transcription from promoters formed by multimeric HNF1 binding sites when added to extracts naturally lacking this transcription factor, such as spleen extracts (Frain *et al.*, 1989; Lichtsteiner and Schibler, 1989; Ryffel *et al.*, 1989; Nicosia *et al.*, 1990).

We report here the identification of two HNF-1 binding sites in the first 200 bp of the human CRP promoter. One is the previously characterized site β in APRE2, the other (defined γ) is located within APRE1. Both sequences, although highly divergent from the proposed consensus sequence (Frain *et al.*, 1989), are able to interact with native and recombinant HNF1. Recombinant HNF1 *trans*-activates the CRP promoter *in vivo* in Hep3B cells. Our data suggest that two HNF-1 molecules bound to different sites act synergistically to activate transcription.

Results

The β element of the CRP promoter is a binding site for HNF1

We have previously shown that the APRE2 of the CRP promoter contains the binding site for two nuclear proteins. One of these sites, named β, interacts with a constitutive protein present in the nuclei of hepatic cells but not in HeLa cells (Majello *et al.*, 1990). A sequence comparison shows a good degree of homology (76.5%) between the β element and the proposed consensus sequence for HNF-1 (Frain *et al.*, 1989) (Figure 1). It should be noted, however, that the two invariant T residues at positions 4 and 5 are substituted by two As.

Gel retardation assays with nuclear extracts from human hepatoma Hep3B cells were performed to analyse the possible binding of HNF1 to the CRP β sequence. The probes were double stranded oligonucleotides reproducing the CRP β sequence (CRP—β) (Majello *et al.*, 1990) and

CONSENSUS HNF1	TGGTTAATNATTAACAA C G	
HUMAN ALBUMIN (PE)	TAGTTAATAATCTACAA	(-65 to -49)
HUMAN CRP (β)	TGGAAAATTATTTACAT	(-74 to -58)
HUMAN CRP (γ)	TGGTTATTTATTACAAA A	(-151 to -168)

Fig. 1. Comparison of CRP β and γ sequences with consensus binding site for HNF-1/LF-B1. The consensus for HNF-1/LF-B1 and the human albumin (PE) sequence are from Frain *et al.* (1989). Underlined are nucleotides which diverge from the consensus.

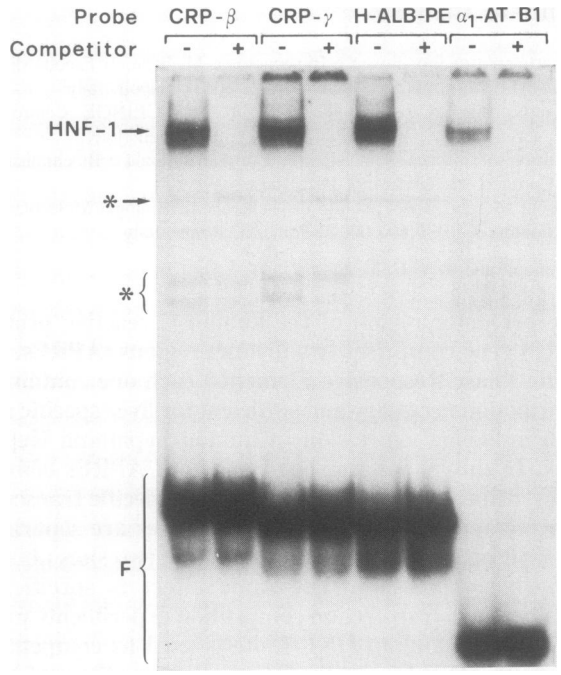

Fig. 2. CRP β and γ give with Hep3B extracts the same pattern of gel retardation as canonical HNF-1 binding sites. Gel mobility shift assays were carried out as described in Materials and methods section. The unspecific bindings are indicated by asterisks. Nuclear extract concentration was adjusted in order to give comparable band-shift intensities. Competitions were performed with 100-fold molar excess of cold oligos.

two typical HNF-1 binding sites: the proximal element of human albumin promoter (H-ALB-PE) (Frain *et al.*, 1989) and the LF-B1 site of the human α1-antitrypsin promoter (α1-AT-B1) (Hardon *et al.*, 1988). These three probes were used in combined cross competition experiments. In some control experiments we used as cold competitors two mutants of the CRP β site (CRP-β Mut-H and Mut-D), the CRP site α (CRP-α) or a sequence of the haptoglobin promoter which shares homology with part of the β sequence and with the promoter of other IL-6 induced genes (HP-111) (Majello *et al.*, 1990).

In a preliminary set of experiments the three probes incubated with Hep3B extracts gave the same pattern of gel retardation (Figure 2). Figure 3A shows that the complex obtained with labelled CRP-β was specifically cross-competed by cold H-ALB-PE but not by the haptoglobin gene derived sequence. In the reverse experiment (Figure 3B) the shift obtained with the H-ALB-PE probe was cross-competed by the CRP-β sequence but not by the CRP-α sequence. The combination of these two experiments strongly supports the idea that HNF-1 is capable of binding the CRP β element.

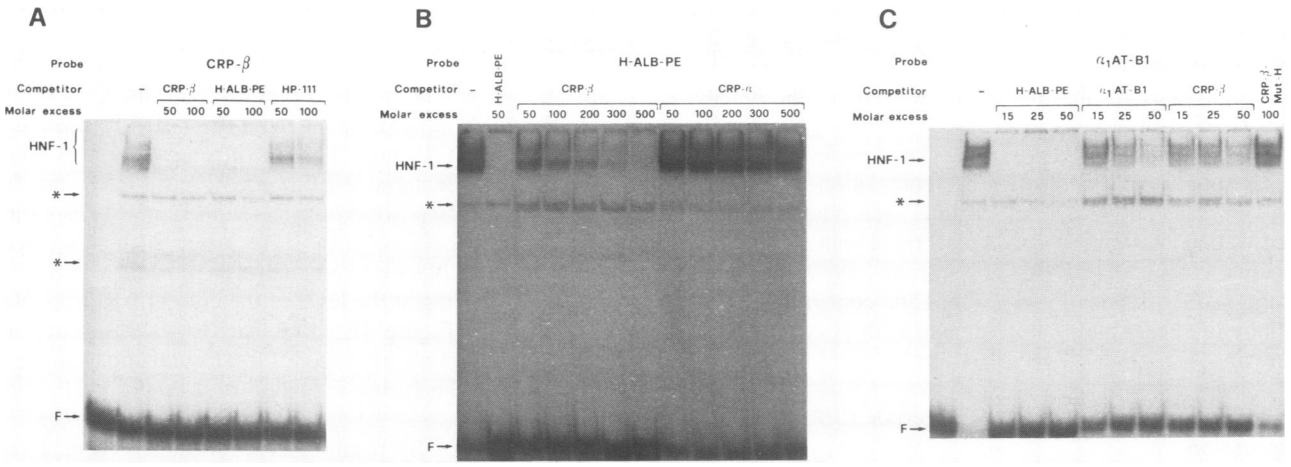

Fig. 3. The protein that binds CRP β is HNF-1. Gel-shift cross-competition experiments were performed with the indicated amounts of cold oligonucleotides (see text) over three labelled probes: CRP-β (panel A); H-ALB-PE (panel B); α1AT-B1 (panel C).

The experiment shown in Figure 3B also shows a difference in the relative affinity of the albumin derived and of the CRP derived sequences for the same factor. HNF-1 appears to bind β 10- to 20-fold less efficiently than to the human albumin PE. This result was confirmed by cross-competition experiments in which lower levels of cold competitors were used (not shown). This finding is not surprising given the previously mentioned divergence of the β site from the consensus HNF-1 sequence, while H-ALB-PE shows a better fit with the consensus (Figure 1). Similar results were obtained using the rat albumin PE (not shown) which was shown to be one of the highest affinity sites for HNF-1 (Cereghini *et al.*, 1988).

The last experiment of this series (Figure 3C) shows how the shift obtained with the α1-antitrypsin probe can be competed by the albumin, α1-antitrypsin and CRP oligos. Also in this case H-ALB-PE was the best competitor, whereas α1-AT B1 and CRP-β displayed a similar binding affinity. It has to be emphasized that the low competing ability of α1-AT-B1 oligo was not due to a low intrinsic affinity of the α1-antitrypsin B1 site for HNF-1, but to the structure of the oligo used in these studies, where the binding site is strongly asymmetric and the T in position 1 is in a single stranded configuration (see Materials and methods). The experiment in Figure 3C shows that a mutant in CRP-β sequence that abolishes the homology with the HNF1 consensus (CRP-β Mut-H) was not able to compete for the same binding.

Methylation interference assays (not shown) gave a pattern of protection of G residues which is in agreement with the previously established contact points of HNF-1 (Cereghini *et al.*, 1988; Hardon *et al.*, 1988). Nevertheless, this experiment does not allow discrimination between HNF-1 and other recently identified relatives, such as V-HNF-1 (Cereghini *et al.*, 1988).

A second binding site for HNF-1 in the CRP promoter

In a previously published footprinting analysis of the human CRP promoter with nuclear extracts from Hep3B cells, we observed the specific protection of a large region from nucleotides −175 to −133 (Majello *et al.*, 1990). This region corresponds, at least in part, to the functionally characterized APRE1. A closer inspection at its sequence

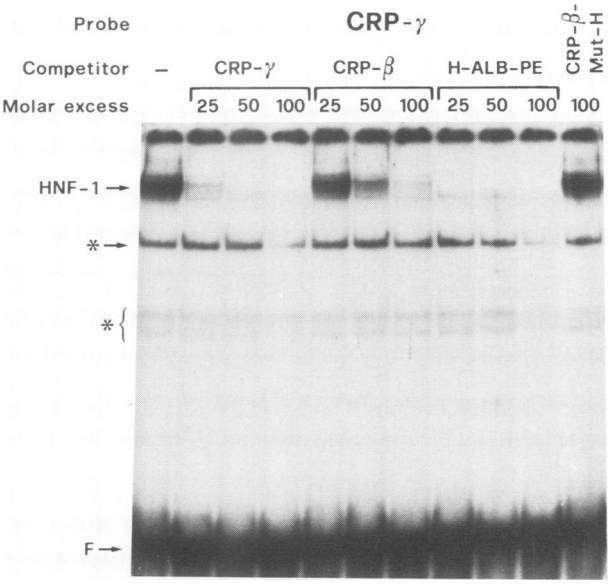

Fig. 4. The protein that binds CRP γ is HNF-1. The end labelled CRP γ oligo was used as probe. Gel-shift cross-competition experiments were performed with the indicated amounts of cold oligonucleotides.

reveals the existence, in the opposite orientation, of another stretch of homology (83.3%) to the consensus binding site for HNF-1 (site γ, Figure 1). Here again, as well as in β, we find divergences from the published consensus: the most significant one is the insertion of an A residue between the two conserved Gs at positions 2 and 3.

We synthesized a ds oligonucleotide (called γ) corresponding to the sequence from −169 to −146 and used it in gel retardation studies with Hep3B nuclear extracts. Also in this case incubation of the probe with crude nuclear extracts from Hep3B cells determined the same pattern of gel retardation as H-ALB-PE and α1-AT-B1 (Figure 2). Cross-competition studies with oligonucleotides β, H-ALB-PE and α1-AT showed that also CRP γ binds HNF1 (Figure 4). This experiment also showed that the binding affinity of site γ for HNF-1 is higher than that of site β, but lower than H-ALB-PE. The specific shift observed with the γ probe was strongly

competed by low concentrations of cold H-ALB-PE competitor, intermediate concentrations of cold γ competitor and high quantities of cold β as competitor. It is possible, therefore, to establish a hierarchy of HNF-1 binding sites with H-ALB-PE > γ > β.

Specific binding of HNF-1 (or related molecules) to site γ was successively confirmed by methylation interference analysis (not shown). Furthermore, gel retardation and methylation interference assays performed with recombinant vaccinia virus LFB1/HNF-1 (Frain *et al.*, 1989) gave identical results with both β and γ oligos and confirmed the low affinity of sites β and γ as compared with H-ALB-PE (not shown).

Mutations of both HNF-1 sites abolish CRP expression

We have previously shown that the two APREs of the CRP promoter act independently from each other as IL-6 inducible elements. This is indicated by the behaviour of two CRP−CAT fusions; the first Δ−94 CRP−CAT carries the first 94 bp of 5′ flanking region (only APRE2), the second Δ−219 CRP−CAT Δ-βα carries the first 219 bp with the internal deletion of nucleotides −94 to −46 (only APRE1). Each of these two constructs is induced by monocyte conditioned medium (MoCM) or IL-6 and, as shown in the previous sections, each one contains a single binding site for HNF-1 (Toniatti *et al.*, 1990).

Base pair substitutions in the β element dramatically affect inducible expression (Majello *et al.*, 1990). We linked −94 w.t. and mutant templates to a different reporter gene, the *Neo* gene (Figure 5A), and performed S1 mapping experiments. The results (Figure 5B) show that transcription of the *Neo* gene was completely abolished by mutations in the β site (D and H), whereas a less dramatic effect was observed with mutations (I and J) in the adjacent downstream sequence (α) which interacts with an IL-6 inducible factor (Majello *et al.*, 1990).

We also deleted the HNF-1 γ site in the context of −219 CRP−*Neo* Δ-βα (Mut-P) (Figure 5A). Also in this case inducible expression was severely impaired (Figure 5C).

A further demonstration of the crucial involvement of HNF-1 in CRP expression comes from competition experiments. Δ−219 CRP−CAT which carries both β and γ HNF-1 sites was cotransfected with increasing amounts of the haptoglobin −111 oligo, of the CRP β oligo or of the H-ALB-PE oligo cloned in multiple copies into the same vector. As one can see in Figure 6 only the latter oligos were able specifically to abolish inducible expression by IL-6, with H-ALB-PE being the most effective competitor, in agreement with its higher affinity for HNF-1.

HNF-1 activity is not modulated by IL-6

The finding of a binding site for HNF-1 in both APREs of the CRP promoter raises the question whether this transcription factor is somehow involved in the IL-6 mediated activation of this gene.

Binding to β and γ sites was not changed both quantitatively and qualitatively in extracts from uninduced and IL-6 induced cells (Majello *et al.*, 1990 and A.Demartis, unpublished). In contrast with this result a multimer of the β site, cloned in the correct orientation in front of the CRP TATA box and linked to the CAT gene gave rise to inducible CAT activity when transfected in Hep3B cells treated with monocyte conditioned medium or with recombinant IL-6 (Majello *et al.*, 1990).

We decided to analyse this issue in greater detail. To this aim we cloned multimetric copies either of the CRP β site or of the albumin proximal element (H-ALB-PE) upstream to the CRP TATA box. These synthetic promoters were linked to the bacterial NEO gene (Figure 7A) and the resulting constructs were transfected in Hep3B cells either uninduced or induced with IL-6. After 24 h RNA was extracted and subjected to S1 analysis to quantitate precisely transcripts starting at the level of the correct CRP cap site. The results in Figure 7B show that IL-6 was not able to induce transcription from any of these synthetic promoters in contrast to the strong induction of a Δ−94 CRP−*Neo* fusion.

Two aspects of this result deserve further comment. First, the promoter constituted by the multimerization of the albumin promoter HNF-1 site showed a high basal level of transcription. This was expected because of the stronger affinity of this site for the cognate transcription factor. The high basal level was not, however, increased by IL-6.

Second, the 4 × β CRP−*Neo* fusion was not transcriptionally activated by IL-6. This is in contrast with the previously reported inducible CAT activity of the analogous 4 × β CRP−CAT fusion. On the basis of what is shown here we must conclude that the CAT activity must be an artifact due to read-through transcription starting in the plasmid.

Recombinant HNF-1 trans-activates the CRP promoter

The transfected CRP promoter is not actively transcribed in Hep 3B cells unless IL-6 is supplemented to the medium. This is in contrast with the presence of HNF-1 in the nuclei and of HNF-1 binding sites in the CRP promoter. We argued that it should be possible to force CRP expression in the absence of IL-6 by increasing the amount of HNF-1 in Hep3B cells. We chose the approach of cotransfecting CRP−CAT fusions with a cDNA for HNF-1 cloned in a suitable expression vector (RSV-HNF-1). All these experiments were performed in the absence of added IL-6.

As one can see in Table I, RSV-HNF-1 was able consistently to *trans*-activate constructs Δ−94 CRP−CAT and Δ−219 CRP−CAT Δ-βα which carry one intact HNF-1 binding site (either γ or β). The level of *trans*-activation when 1 μg of expression vector is used, varies from 5- to ~ 8-fold depending on the construct. Furthermore, CRP promoter mutants in both HNF-1 sites lose their ability to be *trans*-activated (Mut-H, Mut-D and Mut-P).

It has been recently reported that HNF-1 can cooperate functionally with other *trans*-acting factors to promote transcription from the albumin promoter both *in vitro* and *in vivo* (Tronche *et al.*, 1989; Lichtsteiner and Schibler, 1989). We analysed the possibility that interaction between two HNF-1 molecules binding to two distant sites might indeed be able to stimulate synergistically transcription from a natural promoter, the human CRP promoter. Three constructs were used for this analysis: Δ−94 CRP−CAT, Δ−219 CRP−CAT Δ-βα and Δ−219 CRP−CAT. The first two carry only one site (β and γ respectively), in the third both sites are separated by the natural distance of ~ 80 bp. These three constructs were cotransfected with increasing amounts of RSV-HNF-1 plasmid. CAT activity was determined and plotted in Figure 8. The transcriptional

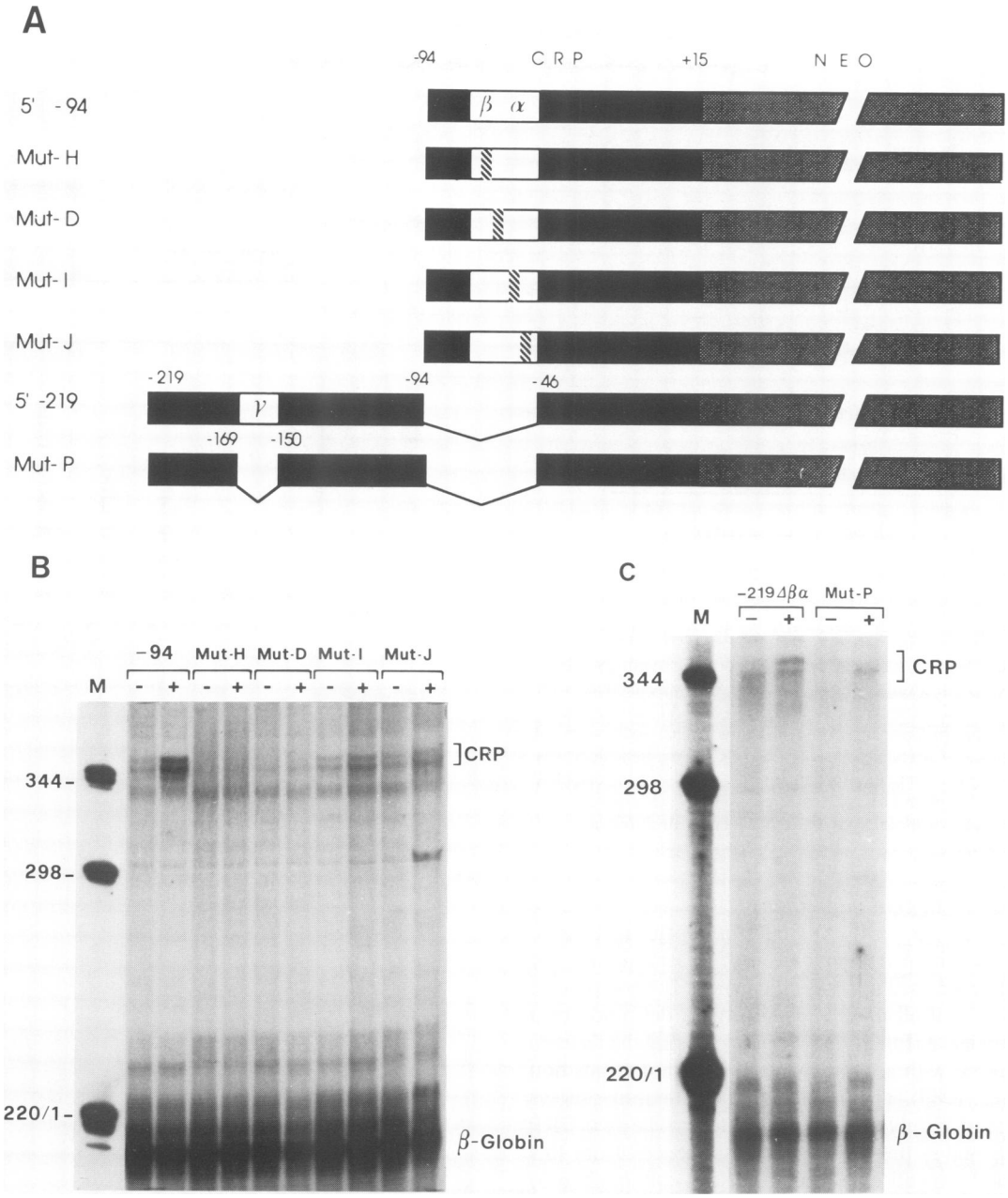

Fig. 5. Mutations of sites β and γ abolish CRP expression. Panel A: schematic representation of the structure of CRP−*Neo* fusions. Black bars represent CRP promoter sequences. Mut-D and Mut-H are base pair substitutions in the β site, Mut-I and Mut-J are base pair substitutions in the α site. The sequence of these four mutants has been reported (Majello *et al.*, 1990). Mut-P is 14 bp deletion in the γ site (see Materials and methods). Panels **B** and **C**: S1 analysis of transcripts generated from CRP−*Neo* constructs. 19 μg of each plasmid, together with 1 μg of control plasmid SV-β-globin were transfected in Hep3B cells. After 12 h cells were washed and either incubated with normal medium (−) or stimulated for 24 h with 500 U of vaccinia recombinant IL-6 (+). For each S1 protection experiment 10 μg of total RNA were used with 2×10^5 c.p.m. of DNA probe. After hybridization and S1 digestion, hybrids were denatured and subsequently electrophoresed on TBE, 7 M urea, 6% polyacrylamide gels. Arrows on the side indicate protected bands of the expected size deriving from transcription starting at the level of CRP cap site (CRP) or of the β-globin gene (β-globin) (Ganter *et al.*, 1989). M: mol. wt size marker, pBR322 digested with *Hin*fI. Gels were exposed for 48 h at −40°C.

activation of Δ−219 CRP−CAT was far higher than the sum of the activity of the other two at any point. Furthermore, the three constructs were highly responsive even at very low concentrations of cotransfected *trans*-activator, but only Δ−219 CRP−CAT did not reach a plateau level at high concentrations of *trans*-activator. This result suggests that two HNF-1 molecules interacting with the same template of distinct sites can functionally cooperate to promote transcription.

Discussion

In this paper we report the identification of two HNF-1 binding sites in the human CRP promoter and present evidence pointing to a crucial involvement of this transcription factor in the liver specific transcription of the IL-6 inducible CRP gene. However, this study allows to draw more general conclusions about the role of HNF-1 in liver specific gene expression.

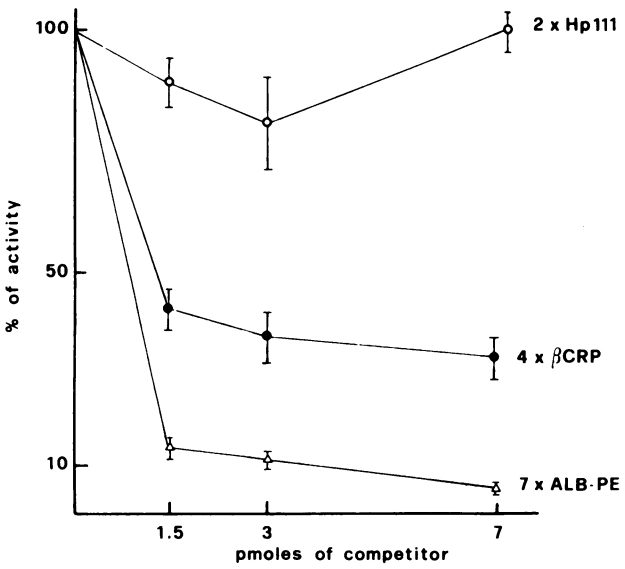

Fig. 6. *In vivo* competition with HNF-1 binding sites abolish CRP expression. Plasmid Δ−219 CRP−CAT was cotransfected with increasing amounts of plasmids carrying multimeric copies of synthetic oligonucleotides. As negative control we used Hp111, a haptoglobin derived sequence bearing a certain degree of homology with the first half of the CRP β site (Majello *et al.*, 1990). Cells were induced with 500 U/ml of vaccinia recombinant IL—6 for 24 h. CAT activity was measured according to Gorman *et al.* (1985). Individual spots were cut and the percentage of acetylated versus total [^{14}C]chloramphenicol was determined by liquid scintillation counting. The activity of uncompeted Δ−219 CRP−CAT plasmid was considered as 100%. The results shown are the mean of three separate experiments with different plasmid preparations.

HNF-1 binds to degenerate sites

HNF-1 has been shown by several laboratories to be able to interact with the promoter region of a large number of constitutively expressed liver specific genes. The comparison of the sequence of all these sites allowed a definition of a consensus sequence (Figure 1) 17 bp long having the features of a palindrome with a dyad symmetry centred at position 9 (Frain *et al.*, 1989). The first half of the palindrome shows the presence of highly conserved residues. The two CRP sequences β and γ present several divergences from this consensus and are still able to bind HNF-1, although at 5- to 20-fold lower affinity than the albumin PE site. This finding is a strong indication of a high degree of tolerance in the interaction of HNF-1 to DNA: several base substitutions in the DNA sequence are tolerated without impairing the binding of the cognate transcription factor. This is probably facilitated by the fact that HNF1 binds DNA as a dimer (Frain *et al.*, 1989) and that divergences from the consensus sequence in one half site can be compensated by binding to the other half-site.

The role of HNF-1 in CRP gene expression

It is intriguing that two atypical HNF-1 sites have been found in the promoter of an inducible gene. Transcription from the CRP promoter in the absence of the appropriate stimulus (IL-6) is very low both *in vivo* and *in vitro*. Moreover, CRP is a liver specific gene and as such has to be under the control of one or more liver specific factor(s). Our results suggest that liver specificity and low basal level of expression are both achieved through a weak interaction with HNF-1. We postulate that the CRP gene, in order to be transcribed at full rate, needs the support of another transcription factor.

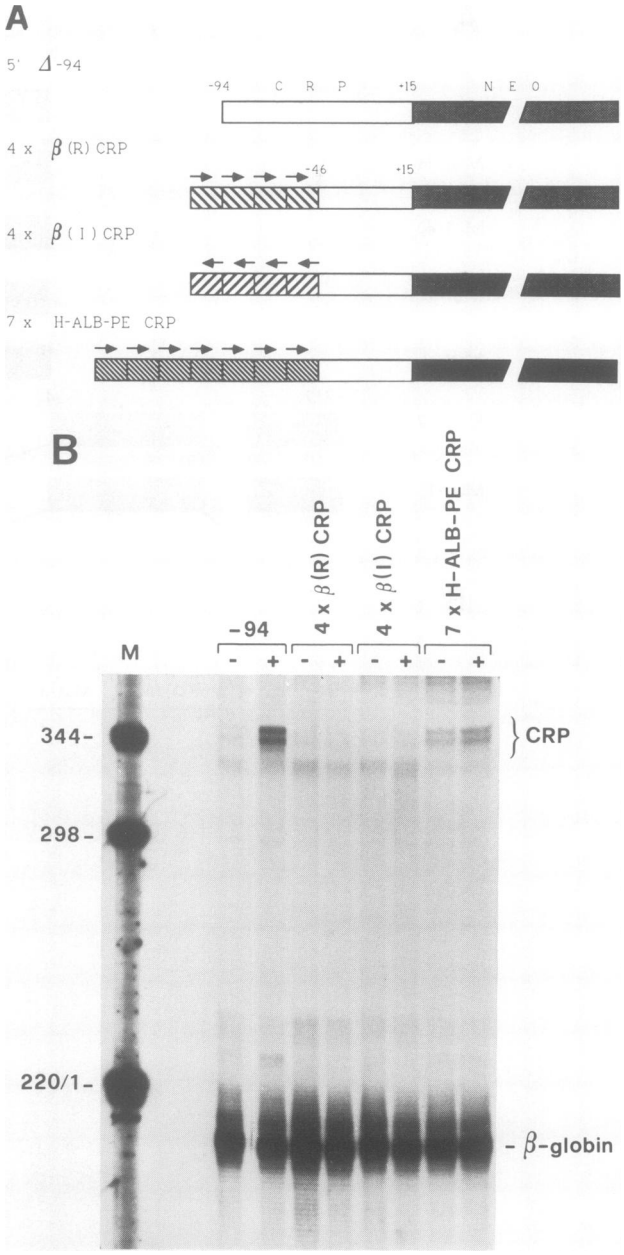

Fig. 7. HNF-1 activity is not modulated by IL-6. Panel **A**: schematic representation of synthetic promoters obtained through multimerization of HNF-1 binding sites linked to the bacterial *Neo* gene. Panel **B**: S1 analysis of transcripts generated from transfections in Hep3B cells in the absence (−) or in the presence (+) of 500 U/ml of vaccinia recombinant IL-6. For details about the S1 analysis see legend to Figure 7 and Materials and methods.

We have recently shown that an IL-6 inducible factor, named H-APF-2/IL-6 DBP, interacts with the promoter region of the CRP gene. In fact, a site for H-APF-2 (α) is located right downstream from the HNF-1 site β (Majello *et al.*, 1990) and mutations of α which abolish interaction with H-APF-2, drastically reduce CRP expression (see Figure 5, mut. I and mut. J). The same factor has been identified as a protein capable of interacting with the promoter of several IL-6 inducible genes (Poli and Cortese, 1989). More recently, a second site for H-APF-2 has been identified also in APRE1 at position −135 to −125 (G.Ciliberto, unpublished). Both APREs, therefore, are characterized by the presence of two sites, one for the IL-6 inducible factor

Table I. The CRP promoter is *trans*-activated *in vivo* by recombinant HNF-1

	% CAP conversion		Fold *trans*-activation
	−	+	
Δ−94 CRP−CAT	0.8	4.7	5.9
Mut-H	0.5	0.5	1.0
MUT-D	0.5	0.5	1.0
MUT-I	1.1	5.5	5.0
MUT-J	0.8	3.8	4.7
Δ−219 CRP−CAT Δ-βα	0.9	7.6	8.4
MUT-P	0.6	0.7	1.2

Different CRP−CAT fusions carrying normal (−94, −219Δ-βα, Mut-I, Mut-J) or mutated (Mut-H, Mut-D, Mut-P) HNF-1 sites were transfected in Hep3B cells with 1 μg of either plasmid pRSV-0 (−) or RSV/HNF-1 (+). All transfections included 1 μg of the reference plasmid RSV-LUC (see Materials and methods). 40 h after transfection, cell extracts were prepared and assayed for CAT activity. Spots were cut and counted and the ratio between incorporated and total counts was determined. Results are expressed as percentage CAP converted to the acetylated form. Values were normalized to the luciferase activity to account for slight differences in transfection efficiency. Fold *trans*-activation indicates the ratio between the activity obtained in the presence and in the absence of RSV/HNF-1.

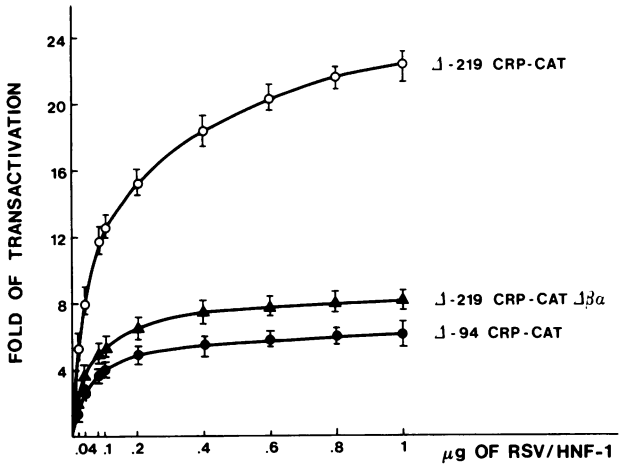

Fig. 8. Synergistic *trans*-activation by HNF-1 binding at two distinct sites. Constructs Δ-219 CRP−CAT (carrying two HNF-1 sites), Δ-219 CRP−CAT Δ-βα and Δ-94 CRP−CAT (carrying a single HNF-1 site) were transfected in Hep3B cells with increasing amounts of RSV/HNF-1 as indicated. The cells were subsequently lysed and assayed for CAT activity. Spots were cut and counted. For each point the ratio between the activity obtained in the presence and in the absence of RSV/HNF-1 was calculated and reported as fold of *trans*-activation. The results shown are the mean of three separate experiments with different plasmid preparations.

H-APF-2/IL-6 DBP, the second for HNF-1. HNF-1 is necessary but not sufficient to ensure active transcription of the CRP gene. The role of HNF-1 is to confer liver specific expression, the role of H-APF-2 to confer IL-6 inducible expression. The weakness of binding of HNF-1 to these sites explains why a synthetic promoter obtained through the multimerization of site β is not constitutively expressed in Hep3B cells, whereas the opposite is true when a strong site (H-ALB-PE) is multimerized. It is possible that in Hep3B cells the number of HNF-1 molecules is just sufficient in normal conditions to drive constitutive expression of genes with high affinity binding sites in their promoters but not of genes, like CRP, with weak binding sites. Upon induction with IL-6, binding of HNF-1 to the CRP promoter could be greatly enhanced by the activation of the other factor H-APF-2/IL-6 DBP which interacts with adjacent sites both in APRE1 and 2. The availability of recombinant H-APF-2/IL-6 DBP will allow a detailed analysis of the cooperative binding of these two factors to DNA both *in vivo* and *in vitro*. Moreover, it will be interesting also to see if other highly inducible liver specific genes show degenerate sites for constitutive liver specific factors.

Our S1 mapping experiments with synthetic promoters demonstrate that the activity of HNF-1 is not influenced by IL-6. In the light of this result, our previous finding of inducible CAT expression of a 4 × β CRP−CAT construct must be interpreted as an artifact due to spurious transcription starting somewhere else in the plasmid and giving rise to a CAT translatable mRNA. We tend to exclude the possibility that the CRP promoter β and γ sites interact with a different protein, with the same binding specificity of HNF-1, but with different functions, such as those of a negative regulator. First, the retarded complex obtained with the β and γ oligos is identical to that obtained with other binding sites, which act as positive elements in the regulation of the albumin and of the α1-antitrypsin genes. Second, the methylation interference pattern with recombinant HNF-1 is identical to that obtained with crude Hep3B nuclear extracts (not shown). Third, we never observed that mutations in the β and γ sites resulted in higher levels of constitutive expression, which would be expected if the protein that binds to these sites was a negative regulator.

Transactivation by HNF-1

Recombinant LF-B1/HNF-1 has been shown to act as a transcriptional activator. VV-HNF-1 confers on spleen extracts the ability to promote transcription from an HNF-1/B1−TK fusion (Frain *et al.*, 1989). In this paper we show that recombinant HNF-1 is also acting *in vivo* as a positive transcriptional activator. It is interesting that this effect can be demonstrated in cells of hepatic origin, which are the natural environment for this transcription factor, because of the specific properties of the HNF-1 sites of the CRP promoter: weak binding sites which are probably not bound and therefore not activated by endogenous HNF-1. The *trans*-activating effect is specific because it is not observed with templates carrying mutated HNF-1 binding sites.

Many different eukaryotic transcriptional activators are able to activate gene expression cooperatively (Giniger and Ptashne 1987; Schüle *et al.*, 1988; Sträle *et al.*, 1988; Kakidani and Ptashne, 1988; Lin *et al.*, 1989; Courey *et al.*, 1989; Carey *et al.*, 1990). An interesting property of HNF-1 is its ability to *trans*-activate synergistically the CRP promoter, where two binding sites are present but separated by ~80 bp. Cooperative *trans*-activation has been recently shown for transcription factor Sp1. In this case, synergism has been demonstrated between a single GC box in the promoter of the TK gene and a six GC repeat cloned downstream of the reporter CAT gene (Courey *et al.*, 1989). In our case synergism is observed with a natural promoter but the degree of cooperativity that we observe is lower than that previously observed for Sp1. This might simply be due to a difference in the architecture of the two reporter constructs used in the different studies. In our case both HNF-1 sites are proximal to the CAP site and therefore the level of *trans*-activation of both constructs carrying a single site is already significant.

The functional cooperation between two HNF-1 molecules is of interest not only because it reveals an interesting property of this *trans*-activator but also because this mechanism might be operating in the activation of CRP transcription. The molecular mechanisms responsible for synergism are not known. In the case of Sp1 synergistic activation is thought to depend on the formation of multimeric forms of the transcription factor, with the generation of a surface that interacts with the transcription apparatus more efficiently than uncomplexed factor. In this case a DNA binding deficient mutant that retains the activating glutamine-rich domain, can interact with a proximally bound Sp1 to superactivate transcription (Courey *et al.*, 1989). In our case plateau levels of *trans*-activation are reached at lower amounts of recombinant HNF-1 for plasmids carrying a single site as compared with the intact promoter. A possible explanation for this finding is that two HNF-1 molecules bound to the CRP promoter are able to attract additional molecules *via* protein−protein interactions. HNF-1 already binds DNA as a dimer and the dimerizing domain has been recently mapped to the amino-terminus of the molecule (Nicosia *et al.*, 1990). However, it will be interesting to see if additional signals for multimer formation are present also in HNF-1, as for Sp1, and therefore if also for HNF-1 superactivation can occur. The activating domain of Sp1 is characterized by a high content of glutamines. HNF-1 does not have a glutamine rich domain. *In vitro* studies with HNF-1 have led to the identification of a split activating region constituted by a serine-rich C-terminal subdomain and a proline-rich more central subdomain (Nicosia *et al.*, 1990). Dissection analysis of HNF-1 in Hep3B will allow to establish if one or the other, or both, subdomains are responsible for synergistic activation of gene expression.

Materials and methods

Nuclear extracts preparation and gel retardation assays

Nuclear extracts from monolayer cells were obtained essentially as described by Lee *et al.* (1988). Binding reactions for bandshift assays used a reaction mix consisting of 10 mM HEPES pH 7.9, 30 mM KCl, 5 mM $MgCl_2$, 5 mM spermidine, 1 mM NaP_i pH 7.2, 0.1 mM EDTA, 0.5 mM DTT, 7% glycerol, 3 μg of poly(dI-dC) and 0.5−1 ng of ^{32}P-5′-labelled ds oligonucleotide (~20 000 c.p.m.). For competition experiments the indicated amount of cold oligonucleotides were added to the mixture. Proteins (4−6 μg) were added to the reaction mix. After 10 mm in ice the samples were directly loaded on 6% polyacrylamide gels (acrylamide: bis-acrylamide 29:1) 0.25 × TBE and electrophoresed at 10 V/cm at 22°C. The gels were dried and subjected to autoradiography (Kodax Xar-5) for 12 h at −40°C.

Plasmid construction and site directed mutagenesis

Most of the CRP−CAT fusions have been described elsewhere (Arcone *et al.*, 1988; Ganter *et al.*, 1989; Majello *et al.*, 1990; Toniatti *et al.*, 1990). Mut-P was obtained by site directed mutagenesis of the previously characterized mutant −219 CRP Δ-βα (Toniatti *et al.*, 1990). Both −219 Δ-βα and Mut-P were cloned as *Eco*RI−*Hin*dIII fragments in front of the bacterial *Neo* gene in plasmid pEMBL-0-Neo (Ganter *et al.*, 1989). The −94 CPR−*Neo* and −46 CRP−Neo constructs have been previously described (Ganter *et al.*, 1989). Similarly, 4 × β(R)CRP−*Neo* and 4 × β(I)CRP−*Neo* were constructed by transferring the promoter regions from the corresponding CAT fusions as *Eco*RI−*Hin*dIII fragments into pEMBL-0-Neo. The 7 × Alb CPR−*Neo* construct was created in two steps. An ALB SV CAT construct containing seven copies in tandem of the H-ALB-PE site cloned in front of the SV40 early promoter−CAT gene was generated (A.Nicosia and P.Monaci, unpublished). In this plasmid the SV40−CAT region was replaced by the −42 CRP−CAT region from the CRP−CAT K mutant (Majello *et al.*, 1990) to give rise to 7 × Alb CRP−CAT. From this the promoter region was released as an *Eco*RI−*Hin*dIII fragment and cloned upstream of the *Neo* coding region in plasmid pEMBL-0-Neo to obtain plasmid 7 × Alb CRP−*Neo*.

The plasmid RSV-HNF-1 was constructed by inserting an *Hinc*II−*Bam*HI fragment from plasmid PB1.2 (Nicosia *et al.*, 1990), corresponding to the DNA segment from nucleotide 65 to 3311 of LF-B1 cDNA (Frain *et al.*, 1989), into expression vector PGM4 (De Simone, unpublished) under the control of the RSV LTR promoter.

Transfections and CAT assays

In vitro cultures and CaP_i transfections of human hepatoma Hep3B cells were performed as described (Arcone *et al.* 1988). For cotransfection competition experiments cells were plated on 5 cm diameter dishes at the density of 10^4/cm^2 and transfected with 2 μg (0.5 pmol) of 5′ Δ−219 CRP−CAT and with increasing amounts (1.5, 3, 4.5 and 7 pmol) of competitor plasmid. The total amount of transfected DNA was kept constant (20 μg) by the addition of carrier DNA (plasmid pEMBL131$^+$) (Dente and Cortese, 1987).

Calcium phosphate precipitates were removed after 15 h and cells were induced for 24 h with recombinant vaccinia virus derived human IL-6 (gift of H.Stunnenberg) at the concentration of 500 U/ml for 24 h.

Cell extracts and CAT assays were performed according to Gorman *et al.* (1985).

Trans-activations were carried out by the cotransfection of 8 μg of reporter plasmid, 1 μg of plasmid RSV-HNF-1 (or carrier plasmid pRSV-0 as control) and 1 μg of RSV-LUC plasmid as internal reference. Cell lysates were prepared 40 h after transfection an analysed for CAT activity. Luciferase activity was determined to control for transfection efficiency as previously described (de Wet, 1987). For the experiments of synergistic *trans*-activation 9 μg of reporter plasmid were cotransfected with the indicated amounts of RSV-HNF-1. Carrier plasmid (pEMBL131$^+$) was used to maintain constant the DNA concentration (10 μg/3 × 10^5 cells).

SI mapping experiments

RNA from transfected Hep3B cells was extracted with the guanidine thiocyanate method (Chirgwin *et al.*, 1979). S1 analyses of transcripts generated from CRP−Neo constructs were performed as described (Ganter *et al.*, 1989). Uniformly labelled DNA probes were obtained by *in vitro* elongation on SS DNA M13 templates (Arcone *et al.*, 1988).

Oligonucleotides

Sequence of oligonucleotides used in this work:

β	5′-TCGAGAAGCAATGTTGGAAAATTATTTACATC-3′
	3′-CTTCGTTACAACCTTTTAATAAATGTAGAGCT-5′
H-ALB-PE	5′-GGGATGAGTCTAGTTAATAATCTACAATT-3′
	3′-TACTCAGATCAATTATTAGATGTTAACCC-5
α1-AT-B1	5′-CCTTGGTTAATATTCACC-3′
	3′-CCAATTATAAGTGGAAGG-5′
γ	5′-TCGAGGTTTGTAATAAATAACTCACATTGC-3′
	3′-CCAAACATTATTTATTGAGTGTAACGAGCTG-5′
α	5′-TCGAGCATAGTGGCGCAAACTCCCTTACTGC-3′
	3′-CGTATCACCGCGTTTGAGGGAATGACGAGCT-5′
Hp111	5′-GATCCGTCACTATCTTTTCCAGTAG-3′
	3′-GCAGTGATAGAAAAGGTCATCCTAG-5′
mut H	5′-CAATGTTGGAGATATCTTTACATAC-3′
	3′GTTACAACCTCTATAGAAATGTATG-5′

For oligos β, γ, H-ALB-PE and α1-AT-B1 underlined sequences correspond to the HNF-1 site.

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