The human haptoglobin gene: transcriptional regulation during development and acute phase induction

Salvatore Oliviero¹, Gianni Morrone and Riccardo Cortese

EMBL, Meyerhofstrasse 1, 6900 Heidelberg, FRG, and Istituto di Scienze Biochimiche, Università di Napoli, Naples, Italy

¹On leave of absence from Istituto di Genetica Medica, University of Turin, Italy

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Haptoglobin is a plasma protein scarcely present in fetal but abundant in adult serum, where it is present at a concentration of ~150 mg/100 ml. In this paper we show by run-on experiments that the haptoglobin (Hp) gene is actively transcribed in adult but not in fetal liver nuclei. Studies with established cell lines indicate that the Hp gene is expressed in the hepatoma cells HepG2 but not in the hepatoma cell line Hep3B nor in HeLa cells. Plasmids carrying various segments of the 5' flanking region of the Hp gene fused to the chloramphenicol acetyl transferase (CAT) gene direct CAT transcription when introduced into HepG2 but are inactive in Hep3B and in HeLa cells, thus behaving like the resident chromosomal Hp gene. Deletion analysis defines a region, upstream to the transcription initiation site, essential for cell-specific expression. The Hp gene is induced in Hep3B cells by treatment with supernatant from LPS-stimulated monocytes (SMS), in a manner mimicking the acute phase reaction. We characterize the DNA segment necessary and sufficient for cell-specific expression of the Hp-CAT constructions in HepG2 and show that the same segment is also sufficient for acute phase induction in Hep3B.

Key words: haptoglobin gene/transcription/cell-specific expression/deletion analysis

Introduction

Haptoglobin (Hp) is a tetrachain ($\alpha_2\beta_2$) glycoprotein synthesized in the liver and secreted into the plasma. The main function of Hp is believed to be the binding to hemoglobin after haemolysis, to prevent kidney damage and loss of iron through urinary excretion (for review see Bowman and Kurosky, 1982). Hp is found in very low concentrations in fetal plasma, whereas its levels in the adult are ~150 mg/ml. It is an acute phase reactant (Kushner *et al.*, 1981; Bowmann and Kurosky, 1982). The acute phase reaction is a change in the concentration of several plasmaproteins, in general synthesized in the liver, in response to acute infection, inflammation and other pathological states. Little is known about the mechanisms responsible for the acute phase reaction. There is increasing evidence that the initial signal derives from macrophages which synthesize and secrete a factor capable of inducing, in hepatocytes, some of the reactions typical of the acute phase (Richtie and Fuller, 1983; Baumann *et al.*, 1986a,b). Thus regulation of the expression of the Hp gene occurs at least at three levels: (i) developmental control, responsible for the lack of expression in fetal liver; (ii) tissue-specific control, responsible for the selectivity of the expression of the

gene in the hepatocyte; (iii) modulation of its expression during the acute phase reaction. The study of Hp gene expression and the establishment of an experimental system suitable for the elucidation of the molecular mechanisms for these various phenomena are the object of this paper.

The Hp locus, located on chromosome 16 (Robson *et al.*, 1969; Simmers *et al.*, 1986), has been the subject of many studies in human genetics: it shows an unusual polymorphism involving duplication, and, rarely, also triplication of parts of the coding region (Maeda *et al.*, 1984; Oliviero *et al.*, 1985a). Human Hp cDNA (Raugei *et al.*, 1983; van der Straten, 1983; Yang *et al.*, 1983) and human Hp genomic segments (Bensi *et al.*, 1985; Maeda, 1985) have been cloned and sequenced. Analysis of the genomic clones has revealed the existence of a second gene, called Hpr, which is adjacent to the haptoglobin (Hp) gene (Maeda *et al.*, 1984; Oliviero *et al.*, 1985b). The sequences of Hp and Hpr are highly homologous, although colinearity is interrupted by an Alu sequence inserted in the 5' flanking region and by a retrovirus-like sequence inserted in the first intron of Hpr (Bensi *et al.*, Maeda, 1985). Hpr does not have any of the features usually associated with pseudogenes, nevertheless it is not expressed in human liver (Bensi *et al.*, 1985).

Here we characterize the expression of Hp [*Hp*² and *Hp*¹ alleles have the same 5' flanking sequence (Bensi *et al.*, 1985; Maeda, 1985)] in fetal and adult human liver, as well as in hepatoma cell lines. The results indicate that a short DNA segment of the 5' flanking region of Hp (from position -186) contains sufficient information for tissue-specific expression and transcriptional activation by acute phase stimuli.

Results

Developmental regulation of the Hp gene occurs at the level of transcription

It has been known for a long time that the concentration of Hp in fetal blood is considerably lower than in adult plasma (Gitlin and Gitlin, 1975). We have previously shown that there are corresponding lower levels of Hp mRNA in fetal liver (Bensi *et al.*, 1985). We now show, by nuclear run-on experiments, that the difference in the steady state levels of mRNA between fetal and adult liver corresponds to a difference in transcriptional rate. In the experiment reported in Figure 1 we hybridized the ³²P-labelled RNA from adult and fetal liver nuclei to various DNA probes. The intensity of the spots of the autoradiograph was quantified by two-dimensional laser densitometry and the values are reported in the table. The Hp gene is transcribed 47 times more in adult than in fetal liver nuclei, compared to a 14-fold difference in the case of the albumin gene, and 16-fold in the case of the hemopexin gene. As expected, the gamma-globin gene is efficiently transcribed only in the fetal liver. The Hpr-specific probe is a 1.8 kb *Bam*HI–*Eco*RI segment from the first intron: the data show that Hpr transcription is not detectable in fetal or adult liver. We have not investigated the reasons for the lack of expression of the Hpr gene further.

Fig. 1. Nuclear transcription in fetal and adult liver cells. Nuclei were prepared from fetal (upper panel) and adult liver (lower panel). The ^{32}P -labelled transcripts were hybridized to pUC19 (pUC), haptoglobin (Hp), haptoglobin-related gene (Hpr), albumin (Alb), γ -globin (γ -Glo) and hemopexin (Hpx) DNAs, immobilized on nitrocellulose filters. In the table the transcriptional ratio between adult and fetus is shown.

Transcription of the Hp gene in the human hepatoma cell lines HepG2 and Hep3B

Human hepatoma cell lines Hep3B and HepG2 have been extensively characterized (Knowles *et al.*, 1981). Both cell lines contain mRNA coding for several plasma proteins including albumin, α_1 -antitrypsin and retinol binding protein (Ciliberto *et al.*, 1985; D'Onofrio *et al.*, 1985). In contrast, Hp and α_1 -acid glycoprotein mRNAs are only present in HepG2. The absence of Hp mRNA in Hep3B could either be trivial, for instance being due to a mutation in the gene which occurred during the establishment of the cell line, or it could be related to the control of gene expression. Southern blot analysis does not reveal any difference between the structure of the Hp locus in Hep3B and HepG2 except that the former derived from an Hp^2 homozygous individual and the latter from an Hp locus heterozygote (Hp^1-Hp^2) (Oliviero, unpublished observations).

The difference in Hp (Figure 2) and AGP (Dente *et al.*, submitted) gene expression between Hep3B and HepG2 mimics the difference between fetal and adult liver. It is therefore possible that the two hepatoma cell lines have stabilized their phenotype

Fig. 2. Northern analysis of HpmRNA in different cell lines and human liver. 10 μg of total cytoplasmic RNAs from cultured cells (lanes 1–3) and 2 μg from human liver (lane 4) were denatured, fractionated on 1.2% agarose gel, transferred onto nitrocellulose filters and hybridized to labelled Hp cDNA. 28S and 18S ribosomal RNAs are indicated.

Fig. 3. CAT activity in extracts of transfected HepG2, Hep3B and HeLa cells. **Upper section:** schematic map of the Hp-Hpr genomic region. Coding regions for Hp and the equivalent regions of Hpr are indicated by solid black boxes for the α -chain and by striped boxes for the β -chain. The open white box indicates the retrovirus-like sequence inserted in the Hpr gene. The 5' flanking region of both genes have been mutagenized and fused in front of the CAT gene as described in the Materials and methods section. **Lower section:** Histogram indicating the CAT activity of cells transfected with Hp-CAT, Hpr-CAT, RBP1-CAT and pEMBL-CAT; pSV2CAT activity is referred to as 100%.

at two different stages of hepatocyte maturation, as a consequence of the process of transformation.

Hp- and Hpr-CAT fusion are transcribed in a cell-specific manner

Expression of the Hp gene is cell-specific. We have investigated the pattern of expression of cloned DNA segments deriving from the Hp gene to map the region containing the information for cell-specific expression. The strategy used in these experiments

Fig. 4. S1 mapping of transcripts from transfected cells. 20 μ g of total cytoplasmic RNA were extracted 36 h after transfection, hybridized to uniformly labelled probes, treated with S1 nuclease, fractionated by gel electrophoresis under denaturing conditions and autoradiographed as described (see Materials and methods). The size of the DNA probes protected from S1 nuclease digestion is indicated in Table I. RBP indicates the RBP-1-CAT transcript (D'Onofrio *et al.*, 1985). β -Glo indicates the two protected bands of the rabbit β -globin gene transcript (Wasylyk *et al.*, 1984) (Table I).

is similar to that used for the study of other liver-specific genes (Ciliberto *et al.*, 1985; D'Onofrio *et al.*, 1987): parts of the 5' flanking region of the Hp gene were fused to the coding region of the bacterial chloramphenicol acetyl transferase (CAT) gene and the resulting constructions were introduced into cultured cells by calcium phosphate precipitation; gene expression was monitored indirectly by measuring CAT enzyme activity and transcript levels were measured by S1 mapping. In a parallel series of experiments we have constructed CAT fusion plasmids using the 5' flanking region of the Hpr gene, which is highly homologous to the Hp gene in the first 183 bp from the cap site, and totally divergent further upstream.

The longest DNA segments from the 5' flanking region of Hp and Hpr used for the CAT fusions were of 1050 and 1120 bp respectively; the corresponding plasmids are called Hp-CAT and Hpr-CAT. In Figure 3, we show the results of a transfection experiment using as recipients the two hepatoma cell lines, HepG2 and Hep3B, and HeLa cells. Both Hp-CAT and Hpr-CAT are expressed only in HepG2. Neither constructions are expressed in HeLa cells, indicating that the 5' flanking region of the corresponding gene contains cell-specific elements. They are not expressed in Hep3B, behaving, in this respect, like the resident Hp gene. It is therefore likely that the differential activity of the chromosomal Hp gene in HepG2 and Hep3B results from different levels of trans-acting transcriptional factors.

Identification of the mimical segment of Hp and Hpr DNA containing sufficient information for cell-specific expression

We have constructed a series of *Bal*31 deletion mutants in the 5'-flanking region of the Hp and Hpr genes and fused them to the CAT coding sequence. Figure 4 shows the transcriptional analysis, by S1 mapping, of the total RNA extracted 36 h after transfection of HepG2, Hep3B and HeLa cells with the various mutant DNAs. In order to have an internal control, in every ex-

Table I. Length of the single-stranded DNA probes and of the expected protected band

m13 constructions	ss probe (bp)	Protected band (bp)
Hp-CAT	467	280
Hpr-CAT	467	280
SV40-CAT	484	309
RBP-1 CAT	469	305
SV40 β -glo	695	201 + 205

periment we co-transfected a plasmid carrying the rabbit β -globin gene driven by the SV40 promoter-enhancer sequence (Wasylyk *et al.*, 1984). The results (Figure 4 and Table II) show that all the mutants retaining 186 bp from the cap site are equally transcribed in HepG2 cells while no signal is detectable in Hep3B or HeLa cells. Initiation of transcription occurs at the expected cap site (Table I) (Bensi *et al.*, 1985). A further deletion of only 31 bp, to position -153, strongly reduces transcription also in HepG2. 5' deletions of the Hpr promoter give the same results with a 2.5-fold stronger signal.

It was interesting to establish whether cell-specific expression could be obtained after substituting the Hp or the Hpr promoter with a heterologous and non-cell-specific promoter. For this purpose we constructed 3' deletions of the Hp upstream region from -1020 to -38 and for Hpr from -1090 to -28. These segments were fused upstream to the 21 bp repeats of the SV40 promoter in the plasmid puc19CAT. Transcription from these plasmids was studied by S1 mapping and the results are shown in Figure 4. 3' Δ -38 and 3' Δ -28 are transcribed only in HepG2 cells and initiation of transcription occurs at the expected site in the SV40 promoter (Table I). Deletion mutants up to -120 for Hp and to -98 for Hpr from the 3' are inactive, showing that, in these constructions, a sequence essential for transcription has been

Fig. 5. DNase I hypersensitive site in the Hp gene. Purified nuclei were treated with increasing amounts of DNase I, the DNA was then purified and restricted with *Hind*III, electrophoresed on a 1% agarose gel, blotted and hybridized with a plasmid (pHp 5) containing a 1500 bp insert of genomic sequence upstream to the Hp gene. In the lower part of the figure the Hp gene schematic map is depicted. B = *Bam*HI, H = *Hind*III. Black boxes indicate α -chain exons, the dashed box indicates the β -chain exon. The dark line indicates the probe used and the arrow indicates the position of the hypersensitive site.

Fig. 6. Northern analysis of mRNA from Hep3B cells. 10 μ g of total cytoplasmic RNA from cultured Hep3B and HepG2 cells, in the absence of (-) or treated with supernatant from LPS-stimulated monocytes (+) were hybridized to haptoglobin cDNA probe.

deleted. Figure 4 also shows the results obtained with a plasmid called RBP-1, containing the retinol binding protein 5' flanking region fused to the CAT coding sequence, used here as positive control for cell-specific expression because it has been previously shown (D'Onofrio *et al.*, 1985) that it is efficiently expressed both in HepG2 and Hep3B but not in HeLa cells.

The promoters of Hp and Hpr do not contain essential infor-

Table II. CAT mRNA levels, measured by densitometric analysis of the S1 protected bands

Constructs	CAT mRNA relative levels		
	HepG2	Hep3B	HeLa
Hp5' Δ -1020	100	<1	<1
-330	98	<1	<1
-264	105	<1	<1
-220	100	<1	<1
-186	93	<1	<1
-153	5	<1	<1
Hp3' Δ -38	620	6	10
Hp3' -120	3	2	8
Hpr5 Δ -1090	455	<1	<1
-730	450	<1	<1
-183	261	<1	<1
-153	7	<1	<1
Hpr3' Δ -28	1405	10	8
-98	11	7	5
-154	9	8	10
RBP-1	694	810	<1

The levels obtained with Hp-CAT have been set equal to 100.

mation for cell-specific expression in the region downstream from the TATA box since this can be replaced by SV40 promoter without the loss of cell specificity. The 5' and 3' boundaries of the essential region must lie between -186 and -38 of Hp and -183 and -28 of Hpr. The shorter fragments that retain full activity showed that the Hpr promoter is ~ 2.5 -fold stronger than the Hp promoter.

The Hp promoter has a DNase I hypersensitive site in HepG2 and in Hep3B but not in HeLa cells

There is in general a strong correlation between specifically located DNase I hypersensitive sites and active genes (Elgin, 1981; Wu, 1980): in the Hp locus we have observed (Figure 5) a hypersensitive site located within 100 bp of the Hp 5' flanking sequence, upstream to the cap site. This site is present both in Hep3B and in HepG2 but is absent in HeLa cells. This result is an indication that, regardless of whether the Hp gene is transcribed, it has the same 'conformation' in the two hepatoma cell lines and a different one in HeLa cells. It is likely that the DNase I hypersensitive site is a consequence of the presence on the DNA of some, but not all, of the factors necessary for Hp gene transcription.

Stimulation of transcription of the Hp gene in Hep3B cells by treatment with supernatant from LPS stimulated monocytes

Recently Darlington *et al.* (1986) have shown that Hep3B cells respond to several stimuli by drastically changing the mRNA levels of several plasma proteins, mimicking the increases and decreases occurring *in vivo* during the acute phase reaction. Similar studies were done on HepG2 by Baumann *et al.* (1986b). We have studied the behaviour of the Hp gene in stimulated Hep3B cells. As shown in Figure 6, there is a dramatic increase of Hp mRNA after a 48 h treatment of the cells with supernatant from LPS-stimulated monocytes (SMS), using a protocol similar to that used by Darlington *et al.* (1986). In this experiment we have allowed the RNA to migrate for a longer distance when compared to the experiment shown in Figure 2, in order to resolve the two Hp mRNAs present in HepG2 cells. They are

Fig. 7. S1 mapping of RNA from transfected Hep3B cells. Hep3B cells were transfected with the indicated constructions, then split in two dishes; one of them was untreated (–) and the other treated with supernatant from LPS activated monocytes (+). 20 µg of total RNA were hybridized with a uniformly labelled probe, subject to S1 nuclease digestion, electrophoresed under denaturing conditions and autoradiographed. β-Glo indicates the two protected bands of the rabbit globin gene transcript. Arrows indicate the protected bands corresponding to the expected transcripts from various Hp-CAT fusions.

the product of the two alleles Hp^1 (the faster species) and Hp^2 (the slower species). In Hep3B only the slower species is present because these cells are Hp^2 homozygous. We have characterized this phenomenon in some detail and the results will be published elsewhere. During this study we have established that a similar treatment causes a 5- to 10-fold increase of the concentration of haptoglobin mRNA also in HepG2 cells, whereas no effect was obtained in HeLa cells (data not shown).

The effect of SMS was more dramatic in Hep3B and therefore we have chosen these cells for further study. We have investigated whether the expression of the transfected DNAs in Hep3B cells could be induced by SMS. The Hp and Hpr constructions previously described were introduced in Hep3B cells by calcium phosphate precipitation. After 5 h, the cells were split and replated in equal numbers in two dishes, one of which was treated with SMS; 36 h after transfection cells were harvested and analyzed by either CAT assay or S1 mapping.

The results shown in Figure 7 and Table III demonstrate that the 5' flanking regions of both the Hp and Hpr genes contain information sufficient for inducibility by SMS. This phenomenon appears to be specific because neither pSV2CAT, pSV2-globin nor RSV-CAT transcription was enhanced by SMS treatment. Analysis of the 5' and 3' deletion mutants shows that full induction in Hep3B is obtained with the constructions that have been

Table III. CAT activity from transfected Hep3B cells, with (+) or without (–) SMS

Constructs	CAT conversion (%)		
	Hep3B –	Hep3B +	+ / –
Hp5'Δ-186	0.21	6.5	30.9
Hp5'Δ-153	0.34	2.4	7.05
Hp3'Δ-38	0.5	13	26
Hp3'Δ-120	0.15	0.5	3.3
Hpr5'Δ-183	3.1	62	20
Hpr5'Δ-153	0.44	2.4	5.45
Hpr3'Δ-28	0.14	2.04	14.5
Hpr3'Δ-98	0.15	1.3	9.5
Hpr3'Δ-154	0.25	0.25	1
pEMBL-CAT	0.29	0.28	0.96
pUC19-CAT	0.2	0.6	3
pSV ₂ -CAT	73	76	1.04
RSV-CAT	86	82	0.96

shown to be maximally active in uninduced HepG2. It is, however, clear that the constructions 5'Δ-153 (both Hp and Hpr) and 3'Δ-98, which are inactive in HepG2, also retain the capacity of being induced, albeit at a lower level. In all cases, initiation of transcription in activated cells occurs at the normal cap site as determined by S1 mapping (Figure 7 and Table I).

Discussion

In vivo expression of the Hp gene

The pattern of gene expression during liver development is characterized by a number of switches. Several genes are active in fetal liver and continue to be active in the adult life (albumin, α₁-antitrypsin, retinol binding protein, apoA1, etc.). Another class of genes is, in physiological conditions, only active in fetal liver. The best characterized member of this class is the gene coding for α-fetoprotein, whose transcription is switched off at birth (Hammer *et al.*, 1987). Another class of genes is only expressed after birth; this class includes the genes coding for α₁-acid glycoprotein (Dente *et al.*, 1987), haptoglobin, and others. The exact timing of this switch has not been established for most genes, but it is reasonable to think that the various physiological events characteristic of birth, including hormonal stimuli, might be responsible for the observed major changes in the pattern of liver-specific gene expression. Another change in liver gene expression occurs when the organism is in a state of acute infection or inflammation (Kushner, 1982). All these phenomena involve a large number of genes and there must be complex regulatory interactions responsible for the maintenance of the differentiated state of the hepatocyte, which at the same time allow for major changes in the specific pattern of gene expression. If, as is likely in the light of the results presented in this paper, several of the regulatory events occur at the level of transcription, then each liver-specific gene must encode for signals capable of interactions with a combination of *trans*-acting factors; these interactions are responsible for tissue-specific expression and quantitative changes in the rate of transcription during development or pathological states.

Our previous knowledge of Hp gene expression suggested that it could constitute an interesting experimental model for the study of the regulatory phenomena of liver-specific gene expression.

Fig. 8. DNA sequence of the 5' flanking region of Hp and Hpr. The differences between Hp and Hpr are indicated. We also report the region of the α_1 -antitrypsin gene that shows significant homology with Hp and Hpr.

In this paper we show by run-on experiments that the developmental regulation of the Hp gene *in vivo* occurs at the level of transcription: the gene appears to be silent in nuclei from fetal liver, and is efficiently transcribed in nuclei from adult liver. In a separate set of experiments we have shown that the Hp gene is expressed in the human hepatoma cell line HepG2 but not in another hepatoma cell line, Hep3B. In this respect HepG2 cells resemble adult hepatocytes and Hep3B cells behave like fetal hepatocytes. In support of this analogy there are additional independent observations: (i) The gene coding for α_1 -acid glycoprotein, expressed in the adult and not in the fetal liver, is expressed in HepG2 but not in Hep3B. (ii) The adult liver-specific histone Ho is found in HepG2 chromatin but not in Hep3B (Gabrielli *et al.*, 1985). At present the idea that HepG2 and Hep3B represent two physiological states similar to adult and fetal hepatocytes respectively can be considered a reasonable working hypothesis.

Difference between Hp and Hpr

The Hp locus is characterized by the presence of two closely related genes: Hp, coding for haptoglobin, and Hpr, whose sequence is highly homologous to that of Hp, but whose gene product has not been identified. The 5' flanking region of these two genes are very similar in the first 183 bases upstream from the cap site, as shown in Figure 8. Further upstream the DNA sequences totally diverge and in Hpr there is an inserted Alu sequence (Bensi *et al.*, 1985; Maeda, 1985). In the coding region (hypothetical in the case of Hpr because the gene product has not been identified), the exon-intron boundaries are in the same position and there is strong homology in the DNA sequence. The only major difference is the presence of a much longer first intron in Hpr, which contains the remnants of a retroviral insertion (Maeda, 1985). In *in vivo* nuclear run-on experiments we showed that Hpr is not transcribed in either fetal or adult liver. More precisely we showed that there is no transcription elongating up to the middle of the first intron; it is still possible, though rather unlikely, that the promoter is active but there is premature transcriptional termination. On the other hand, *in vivo* transfection experiments indicate that the Hpr promoter is active and cell specific, in fact more active than the Hp promoter. We therefore do not know why the Hpr gene is not transcribed *in vivo* and have not specifically addressed this question in the present study.

Tissue-specific control

Hp is synthesized in the liver. In this paper we show that the 5' flanking regions of Hp and Hpr contain information capable of driving transcription in the hepatoma cell line HepG2 but not Hep3B nor in HeLa cells. We have identified the minimum seg-

ment containing sufficient information for cell-specific transcription. Our data indicate that a DNA sequence found between position -186 (-183 in the case of Hpr) and -38 (-28 in the case of Hpr) contains transcriptional signals capable of activating the SV40 promoter (21 base pair repeats and TATA) specifically in HepG2. Having established that the information for cell-specific expression is contained in a short DNA segment of Hp gene, it is not surprising that a highly homologous DNA segment derived from the 5' flanking region of the Hpr gene contains equivalent information.

It is interesting to note that the liver-specific genes characterized so far differ with respect to the importance of the DNA sequence surrounding the TATA box: Hp and Hpr behave like the α_1 -antitrypsin (Ciliberto *et al.*, 1985) and the ApoA1 gene (Colombo and Cortese, manuscript in preparation): their upstream transcriptional signals activate the SV40 promoter only in hepatoma cell lines. In contrast, the upstream sequences of the retinol binding protein gene (D'Onofrio *et al.*, 1985; Colantuoni *et al.*, 1987) and of the albumin gene (Frain *et al.*, manuscript in preparation) act in a cell-specific manner only with their own or another liver-specific promoter, which cannot be substituted by the SV40 promoter without loss of cell specificity. These results emphasize that more than one mechanism for ensuring tissue specificity of expression has been adopted by liver-specific genes.

In our experimental conditions the plasmid containing 213 (-183/+30) bp from the 5' flanking region of Hpr is expressed 2.5-fold more than a similar construction containing 216 bp from the 5' flanking region of Hp. This difference is presumably due to the few dissimilarities in the DNA sequence between the two genes (Figure 8). Hpr differs from Hp at eight positions within the region under consideration. The most interesting differences are at positions -137 and -135, because these are located in proximity of a conserved region between Hp, Hpr and the α_1 -antitrypsin gene (Figure 8). In this latter gene, mutations in this box eliminate transcription (De Simone, Lundberg and Cortese, unpublished data). In an extension of the present work we will examine this point by site-directed mutagenesis of Hp and Hpr.

Acute phase induction

Recently Darlington *et al.* (1986) have shown that Hep3B cells respond to acute phase stimuli by changing the intracellular mRNA concentration of several genes coding for well characterized acute-phase reactants. We have exploited this observation to examine in more detail the mechanism of the response in the case of Hp. There is no Hp mRNA in Hep3B cells, but the Hp gene contains a DNase I-hypersensitive site in the promoter region, indistinguishable from that found in the active HepG2 gene and which is absent in the Hp gene of HeLa cells. This could mean either than the gene is active but the mRNA is highly unstable, or that the gene is silent. The latter possibility, substantiated by the transfection experiments discussed below, implies that the activation of the Hp gene in hepatic cells occurs in two steps, and that in Hep3B the first step of activation, leading to the appearance of a DNase I-hypersensitive site, has already occurred. Following treatment with SMS, Hep3B cells accumulate Hp mRNA. The transfected Hp and Hpr-CAT fusions, in which the transcript consists mostly of the CAT coding sequence, behave like the endogenous Hp gene showing that SMS treatment causes an increase in the rate of transcription. This suggests that the acute phase reaction involves or even consists of changes in the concentration or activity of transcriptional *trans*-acting factors.

Deletion analysis of these promoters gives further information. It appears that maximal inducibility in Hep3B is obtained with constructs that are active in HepG2. It is also clear that constructs with shorter Hp or Hpr 5' flanking sequences, which are not active in HepG2, still retain the capacity to be induced in Hep3B cells, although the degree of the response is reduced. Combining the results obtained from the 5' and the 3' deletions, we conclude that the region between -152 and -98 in the Hpr 5' flanking region contains information sufficient for inducibility; it could contain a site recognized by an acute-phase induced positive transacting factor. Within this region there is a conserved sequence (Figure 8) present in the inverted orientation between positions -103 and -116 in the gene coding for α_1 -antitrypsin (Ciliberto *et al.*, 1985), also an acute phase reactant (Kushner *et al.*, 1981). It remains to be established whether the remaining DNA sequences preceding and following the region, and which, when present, cause a much higher transcriptional rate in HepG2 and a stronger induction by SMS in Hep3B, contain additional information specific for the response to SMS or simply potentiate it by binding to *trans*-acting factors essential for efficient transcription.

Materials and methods

Cell culture

Human hepatoma cell lines HepG2 and Hep3B (Knowles *et al.*, 1980) and HeLa cells were cultured as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, glutamine (final concentration 5 mM) and penicillin/streptomycin (100 U/ml). Induction was obtained by incubating the cells in the presence of culture medium from LPS stimulated monocytes as described by Darlington *et al.* (1986) except that 10% supernatant was used.

Nuclear run-on transcription

Measurements of transcription in isolated nuclei were essentially as described (Vannice *et al.*, 1984). Nuclei were stored at -80°C in a glycerol buffer (50 mM Hepes pH 8, 1 mM MgCl₂, 0.5 mM DTT, 1 mg/ml BSA, 25% glycerol) without loss of activity. Human fetal nuclei were obtained from fetuses 10–14 weeks old. Five microgrammes of linearized plasmids were spotted onto cellulose filters. The probes utilized were: Hp cDNA (Raugei *et al.*, 1983). A plasmid called Hpr4 containing a 1800 bp *Bam*HI–*Eco*RI insert deriving from the first intron of Hpr (Bensi *et al.*, 1985). Albumin cDNA (Lavareda *et al.*, 1984), kindly provided by M. Frain. For γ -globin we used a plasmid, pHg 1, with a 5.1 kb genomic DNA fragment containing both the G γ and A γ genes (Ottolenghi, unpublished data). For hemopexin we used a plasmid, Hpx11, which contains the complete hemopexin cDNA (Altruda *et al.*, 1985).

Plasmid constructions

Hp and Hpr *Xba*I–*Bgl*II genomic fragments (Raugei *et al.*, 1983) were subcloned into mp12 and mutagenized as previously described (Sollazzo *et al.*, 1985) with the oligo TCTGGTTGGTTCGAAAGGGAGGAC, to create an *Hind*III site at position +30. The *Xba*I–*Hind*III DNA fragments were then subcloned in pUC19. *Bal*31 deletions were performed by incubating the linearized plasmids different times in the presence of *Bal*31 exonuclease. The resulting fragments were subcloned in mp18 or mp19 and sequenced. The *Xba*I–*Hind*III fragments and the 5' deletions were cloned in pEMBL8-CAT (M. Uhlén, in preparation), a plasmid containing the mp8 polylinker in front of the *Hind*III–*Bam*HI CAT cassette from pSV2 CAT (Gorman *et al.*, 1982). 3' deletions were cloned upstream to the SV40 promoter into pUC19CAT. This vector contains the SV40 TATA box and the 21 bp repeats up to position 179 of the SV40 DNA physical map (Tooze, 1980) fused to the *Hind*III–*Bam*HI CAT cassette from pSV2CAT and preceded by a polylinker (Ciliberto *et al.*, in preparation).

DNase I digestion

2×10^7 HepG2, Hep3B or HeLa cells were washed in PBS and then resuspended in 4 ml of buffer A (10 mM Tris pH 7.4, 10 mM NaCl, 3 mM MgCl₂), and homogenized in Dounce with tight pestle (10–15 strokes). The homogenate was layered over 0.3 M sucrose in the same buffer and sedimented at 2000 g at 4°C for 20 min. The nuclei were resuspended in 600 μ l of digestion buffer (15 mM Tris pH 7.5, 3 mM MgCl₂, 0.5 mM DTT, 60 mM KCl, NaCl, 0.25 mM sucrose). All digestions were performed at 37°C for 10 min with 0.5 to 10 units/ml of DNase I (Worthington) and stopped by addition of EDTA (final concentration 25 mM) and 0.5% sodium dodecyl sulphate (SDS). DNA purification, restriction enzyme digestions, Southern blotting and hybridization were carried out as

previously described (Oliviero *et al.*, 1985b), using as probe a ³²P-labelled DNA fragment from the 5' flanking region of the Hp gene, as indicated in Figure 5.

DNA transfection and induction

DNA transfection was performed by the calcium phosphate precipitation technique (Graham and van der Eb, 1973). For induction, cells from each dish were split in two dishes 5 h after transfection, one of which was then treated with medium from activated monocytes. The cells were harvested 36 h after transfection either for the CAT assay (Gorman *et al.*, 1982) or for RNA extraction with the guanidine thiocyanate procedure (Raugei *et al.*, 1983).

S1 mapping

S1 mapping was performed according to Berk and Sharp (1977) as modified by Ciliberto *et al.* (1983). All probes were obtained by *in vitro* elongation on suitable m13 single strand templates. For transcripts from Hp and Hpr-CAT fusions *Sac*I–*Eco*RI segments from Hp- or Hpr-CAT were cloned into mp19. For transcripts from SV2-CAT and the plasmids where the haptoglobin gene promoter was substituted with that of SV40, a *Bam*HI–*Eco*RI segment from SV2-CAT was cloned in mp19. For transcripts from SV40- β -globin a *Bam*HI–*Bam*HI fragment (Wasylyk *et al.*, 1984) was cloned into *Bam*HI site of mp19. For the synthesis of radioactive probes 400 μ g of template single strand phage DNA were incubated with a 25mer sequencing primer with 50 mM NaCl, 10 mM Tris pH 7.4, 10 mM MgSO₄, 1 mM DTT, 100 μ M dGTP, 100 μ M dTTP, 40 μ Ci [α -³²P]dATP and 40 μ Ci [α -³²P]dCTP (sp. act. 3000 Ci/mmol) with 10 units DNA polymerase I large fragment, in a total volume of 20 μ l. After 15 min at room temperature, dATP and dCTP were added to a final concentration of 100 μ M, and the incubation continued for 15 min. The synthesized DNA was subjected to single restriction cut (*Sac*I for Hp and Hpr probes, *Bam*HI for SV40 and *Pst*I for β -globin) and the resulting probes isolated by polyacrylamide electrophoresis under denaturing conditions.

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