Volume 17 Number 3 1989 Nucleic Acids Research

Liver cell specific gene transcription *in vitro*: the promoter elements HP1 and TATA box are necessary and sufficient to generate a liver-specific promoter

Gerhart U.Ryffel, Wilfried Kugler, Ulrike Wagner and Michael Kaling

Kernforschungszentrum Karlsruhe, Institut für Genetik und Toxikologie, Postfach 3640, D-7500 Karlsruhe 1, FRG

Received November 16, 1988; Revised and Accepted January 3, 1989

ABSTRACT

The hepatocyte-specific promoter element HP1, which is present in several genes specifically expressed in the liver, is active in an in vitro transcription system. The liver-specificity is retained in the in vitro system, as the activity is found in extracts of rat liver or hepatoma cells but is absent in an L-cell extract. Mutational analysis identifies HP1 as a 13 bp element: Two point mutations abolish the function of HP1. This inactivation is correlated with a reduced binding affinity of the transcription factors recognizing HP1. Two other mutants, which reduce the activity of HP1, bind the transcription factors with an affinity identical to the wildtype sequence. This suggests that the binding of the transcription factors is not sufficient for activation of HP1 dependent transcription. The function of HP1 depends on the presence of a TATA box within a distance of some 70 bp. Various TATA sequences are functional and no stereospecific alignment between HP1 and the TATA box is required.

INTRODUCTION

Tissue-specific gene expression is largely due to differential transcription of genes. Recent experiments have documented that at least some of this transcriptional selectivity depends on the presence of tissue-specific transcription factors some of which have been identified in in vitro transcription assays (1, 2, 3, 4, 5, 6.). In the case of liver-specific gene transcription we have identified in the Xenopus albumin gene the regulatory sequence HP1 (hepatocyte-specific promoter element) that in transfection experiments confers expression restricted to hepatocytes (7). Since the presence of HP1 increases the transcriptional activity of a promoter in vitro in a nuclear extract from rat liver cells we conclude that transcription factors present in hepatocytes interact with HP1. Based on in vitro transcription experiments and binding assays we were able to show (8) that HP1 is identical to regulatory elements found in mammalian albumin and AFP (α-fetoprotein) genes but also to sequences identified in the rat ß-fibrinogen gene (9) and the human α_1-antitrypsin gene promoter (10). Thus, HP1 represents a promoter element that confers liver-specific expression to several different genes known to be selectively transcribed in hepatocytes.

In the present report we establish that HP1 is a 13 bp element that together with the TATA box is sufficient to form a minimal promoter with liver-specific activity in an in vitro transcription system. Using mutants of HP1 we investigated the relationship between HP1 function and the binding of transcription factors.

MATERIALS AND METHODS

Cell culture and media

Ltk$^-$ cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin (100 U/ml). HepG2 and FTO-2B cells were grown in a 1:1 DMEM/Ham's F-12 medium mixture containing 10% FCS and penicillin/streptomycin (100 U/ml).

Nuclear extract preparation

Rat liver nuclear extract was prepared essentially as described by Gorski et al. (1). Nuclear extracts from cells grown in cell culture were prepared according to a modification of the method of Shapiro et al. (11). All manipulations were done at 0°C. Cells were harvested, washed twice with ice cold PBS, and collected by low spin centrifugation. Cells were resuspended in 5 packed cell volumes (PCV) of HB1 (10 mM HEPES-KOH, pH 7.6; 10 mM KCl; 0.75 mM spermidine; 0.15 mM spermine; 0.1 mM EDTA; 1 mM DTT) and allowed to swell on ice for 10 min. After centrifugation for 10 min at 300xg, cells were resuspended in 2 PCV HB1 and homogenized with 3 strokes using a glass homogenizer. 0.1 Vol of HB2 (67.5% sucrose in HB1) was added, mixed, and the homogenate was centrifuged immediately for 30 sec at 10,000 rpm in Sorvall HB-4 rotor. The total centrifugation time was kept as low as possible (2 to 3 min). Pelleted nuclei were resuspended and lyzed in 3 ml nuclear lysis buffer (NLB) per $1x10^9$ cells. (NLB:18 mM HEPES-KOH, pH 7.6; 0.675 mM spermidine; 0.135 mM spermine; 0.18 mM EGTA; 0.18 mM EDTA; 1.8 mM DTT; 22.5% glycerol; 0.4 M $(NH_4)_2 SO_4$). After shaking for 30 min on ice the viscous lysate was centrifuged for 90 min at 150.000xg. Nuclear proteins were precipitated from the supernatant by adding 0.33g/ml solid $(NH_4)_2 SO_4$. The precipitate was collected by centrifugation at 85.000xg for 20 min and redissolved in dialysis buffer (20 mM HEPES-KOH, pH 7.6; 100 mM KCl; 0.2 mM EGTA; 0.2 mM EDTA; 2 mM DTT; 20% glycerol) at a maximum volume of 1 ml per $1x10^9$ cells. The extract was dialyzed twice for 90 min against 250 ml each of the same buffer, centrifuged briefly to remove the precipitate formed during dialysis and stored in aliquots in liquid nitrogen.

In vitro transcription

In vitro transcription reactions were performed as previously described (6). When using nuclear extracts of cells grown in culture the generated transcripts were treated with 15U RNase T1 for 30 min at 30°C. This incubation degrades G-containing transcripts made due to high concentrations of endogenous GTP in these extracts.

RESULTS

Liver-specific transcription in vitro is mediated by HP1

In the Xenopus 68 kd albumin gene we have defined by transfection experiments a hepatocyte-specific promoter element, HP1, that confers transcriptional activation restricted to hepatocytes. Using nuclear extracts from rat liver we have shown that HP1 can functionally

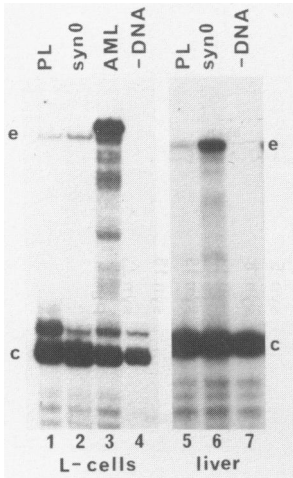

Figure 1: Liver-specific transcription in vitro
Constructs containing the TATA box region of the Xenopus 68 kd albumin gene linked to the -G-cassette were used in in vitro transcription assays with nuclear extracts from L-cells (lanes 1-4) and rat liver (lanes 5-7). The TATA box region was combined either with a polylinker sequence (PL) or HP1 (syn 0) as found in the Xenopus albumin gene (Fig. 7). As control we used either the adeno major late promoter (AML) linked to the -G-cassette (pML(C_2AT) 19) as constructed by Sawadogo and Roeder (32), or no template (-DNA). The signal migrating to a position corresponding to an expected size of 400 nt (e) represents the G-less transcript derived from the promoter to be tested. The signal of 200 nt (c) indicates transcription from the internal control template with the adeno major late promoter linked to a shortened -G-cassette, which was included in all reactions (7).

interact with transcription factors present in liver cells (7). To investigate whether tissue-specificity holds also true for this in vitro assay, we performed in vitro transcription experiments using a nuclear extract from L-cells. In these mouse fibroblasts we have previously shown by transfection experiments that HP1 is not functional (7). To assay the in vitro transcription reactions a -G-cassette of 400 bp was linked to the proximal promoter region of the Xenopus albumin gene (position -67 to -26) containing HP1 and the TATA box (construct syn O-TG in ref. 7). A shortened G-free cassette of about 200 bp under the control of the adenovirus major late promoter was included as an internal control in all reactions. Fig. 1 demonstrates that in an L-cell extract transcripts of 400 bp (e), indicative for transcription that might be driven by HP1, are low. Taking into account the activity of the internal control (c) no significantly higher level of transcription is observed with syn O-TG (lane 2) compared to a promoter in which HP1 is exchanged for a polylinker sequence (lane 1). Testing the same templates with a rat liver nuclear extract we observed high activity of the promoter containing HP1 (lane 6), whereas the activity of the polylinker sequence remained low (lane 5, compare ref. 7). However, the adeno major late promoter showed high activity in both extracts (lane 3 and internal control (c) in all lanes). Inability to increase in vitro transcription by HP1 was also observed using nuclear extracts from MCF-7 cells (data not shown) or from Hela cells (K. Ross, unpublished). Based on these findings we conclude that the liver-specific function of HP1 is maintained in an in vitro transcription system.

Single point mutations introduced into HP1 destroy its function in vitro

To investigate the sequence requirements for a functional HP1, we selectively changed single nucleotides in the regulatory element (Fig. 2a) and analyzed the transcriptional activity of these mutants in a rat liver nuclear extract. The mutants analyzed have previously been

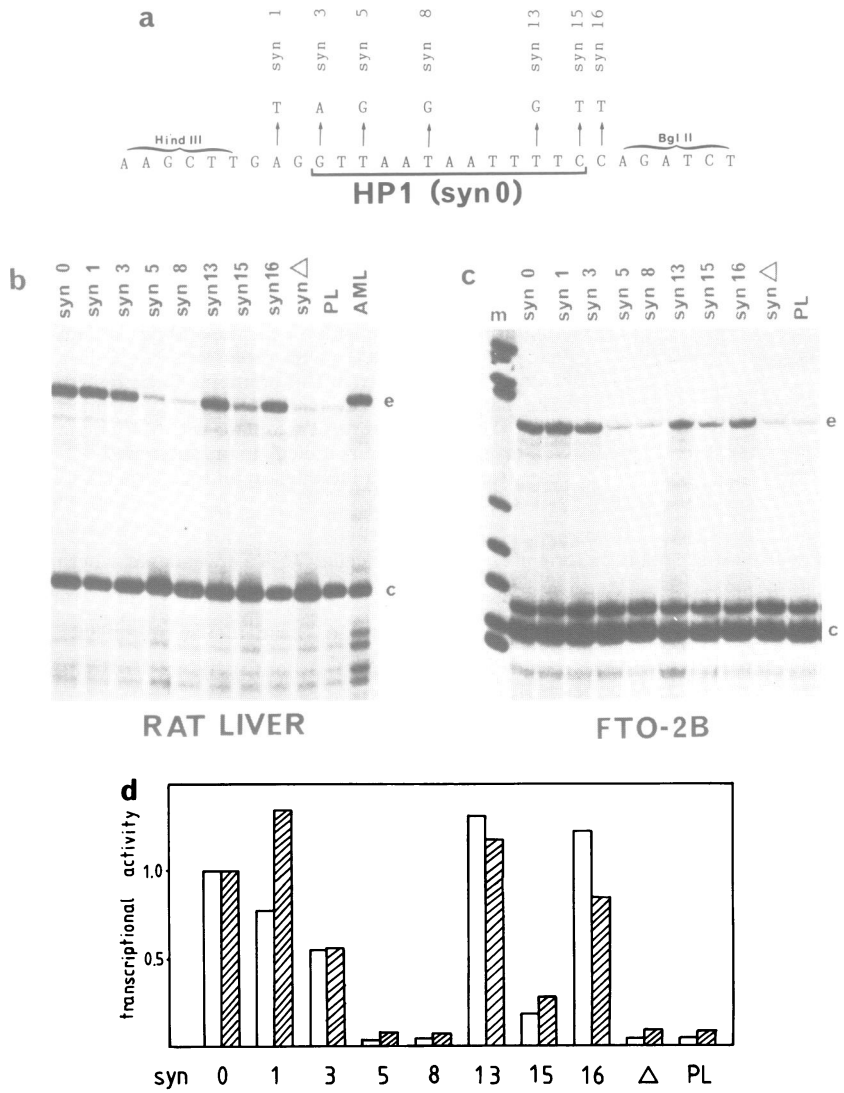

Figure 2: Mutational analysis of HP1 in in vitro transcription with rat liver and rat hepatoma cell extracts

(a) The oligonucleotide containing HP1 of the Xenopus 68 kd albumin gene (syn 0) is given with the single point mutations introduced (7) to generate the indicated mutated HP1. In vitro transcription in nuclear extracts from rat liver (b) and FTO-2B hepatoma cells (c) is illustrated using syn 0-TG (syn 0) or the various HP1 mutant constructs. Syn Δ contains a 6 bp deletion in HP1 (7). Further details are given in Fig. 1. (d) The data of the in vitro transcription in both extracts were quantified by densitometric scanning (open columns: rat liver nuclear extract; stripped columns: FTO-2B nuclear extract). Further details are given in the text.

tested in transfection assays in vivo (7): they were chosen in such a way as to alter specific positions in the presumed regulatory region comparing various albumin and AFP gene promoters of vertebrates. As illustrated in Fig. 2b, two point mutations, syn 5 and syn 8, abolish HP1 function completely. The residual activity retained is as low as the one found with constructs where HP1 has been replaced by a polylinker sequence (lane PL) or where 6 nucleotides of HP1 have been deleted (lane syn Δ, see ref. 7 for details). Two other mutants, syn 3 and syn 15, that constitute the borders of HP1 (see Fig. 2a) partially reduce HP1 activity. To quantify the effects, we measured the transcriptional signals using the activity of the internal control as a standard. Fig. 2d (open columns) summarizes the quantifications of three independent determinations using two different preparations of rat liver nuclear extract. Clearly, syn 3 and syn 15 reduce the function of HP1 two- and four-fold, respectively, compared to the wildtype sequence syn 0, whereas syn 5 and syn 8 show only background activity. The quantification also illustrates that the two point mutations outside of HP1, syn 1 and syn 16, do not alter the in vitro transcription. Most notably, the internal point mutation syn 13 does not significantly alter HP1 specific transcription.

Since HP1 was initially defined by DNA transfection into hepatoma cells (6, 7), we analyzed the same constructs in extracts derived from rat hepatoma FTO-2B cells and human hepatoma HepG2 cells. Since the results obtained with both of these extracts are essentially the same, we only show the data for the FTO-2B extract. A representative example is given in Fig. 2c and the quantified data derived from two independent assays are included in Fig. 2d (stripped bars). From all these assays we conclude that the point mutations behave identical in hepatoma and rat liver nuclear extracts. This suggests that the same transcriptional factors are present in all of these extracts.

Transcription factors bind with reduced affinity to non-functional HP1 mutants

That single point mutations, syn 5 and syn 8, destroy the function of HP1, can be interpreted to indicate either that the transcription factors do not bind to the mutated HP1 or that the interaction does not lead to the formation of a functional initiation complex. To sort out which of these two interpretations is correct, we included the various HP1 oligonucleotides as competitors in the in vitro transcription reaction. Assuming that the transcription factors do not bind to a given HP1 mutant, we would expect this HP1 oligonucleotide to have reduced competition abilities. Based on these considerations we performed in vitro transcription experiments using syn 0-TG containing the wildtype HP1 as template and included for competition synthetic oligonucleotides carrying the mutant HP1 sequences. Fig. 3 illustrates that 10 ng of the wildtype HP1 oligonucleotide (syn 0) are sufficient to get competition whereas mutant oligonucleotide syn 5 is a less efficient competitor and syn 8 has no effect. On the other hand syn 15 has similar competition properties as the wildtype sequence syn 0 (Fig. 3). The specificity of the competition is documented by the fact that the activity of the adeno major late promoter (lower band, c, in Fig. 3) is not affected. Further excess of

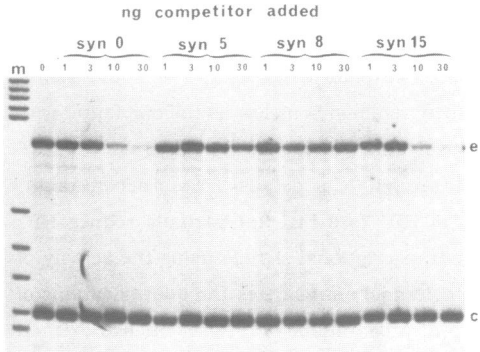

Figure 3: In vitro transcription using various HP1 oligonucleotides as competitors.
Syn 0-TG (135 ng) was used as template for in vitro transcription in a rat liver nuclear extract. As competitors the indicated double stranded oligonucleotides were included in the reaction (see Fig. 2a for nomenclature). 30 ng competitor corresponds to a 100-fold molar excess to the template. Further details see Fig. 1.

competitor oligonucleotides (200ng) reduces specificity: All mutant oligonucleotides except syn Δ that lacks 6 nucleotides of HP1, were able to compete for transcription factors binding to HP1 (data not shown). When quantified (Fig. 5a), the similar competition behaviour of syn 3, syn 15 and syn 0 becomes evident, in contrast to syn 5 and syn 8 which compete much less efficiently. Based on these experiments we assume that mutants syn 5 and syn 8 lead to reduced binding affinity of the transcription factors recognizing HP1. This reduction may explain the observation that syn 5 and syn 8 replacing HP1 in the transcription template syn 0-TG are not functional (see Fig. 2d). The in vitro transcription assays have identified two other point mutations that interfere with HP1 function, although both of these mutants, syn 3 and syn 15, have identical competition properties as the wildtype sequence syn 0 (Fig. 5a). Obviously, in these cases the reduced function cannot be correlated with a corresponding decrease in affinity of the HP1 binding factors to the oligonucleotide.

The HP1 mediated complex leading to transcriptional activation correlates with a protein-oligonucleotide complex

We know from our in vitro binding studies that nuclear proteins specific for liver cells bind to HP1 (7). This interaction can readily be seen in gel retardation assays using the wildtype HP1, syn 0, as labelled oligonucleotide. We analyzed how this complex relates to the one leading to activation in vitro. If both complexes are identical, we would expect them to have the same sensitivity to the addition of competing wildtype and mutant HP1 oligonucleotides in both of the assays. Therefore we performed gel retardation experiments including increasing amounts of the various oligonucleotides as competitors. Three examples are given in Fig. 4: The wildtype sequence syn 0 forms a complex of low mobility with rat liver nuclear extract as previously found for mouse and human hepatoma cell extracts (7). This complex can be specifically competed by the addition of unlabelled syn 0. Using the oligonucleotides syn 5 and syn 8 as competitors, a considerable larger amount has to be present in the reaction to obtain similar competition. In fact, syn 8 competes efficiently only at an input of 200 ng (data

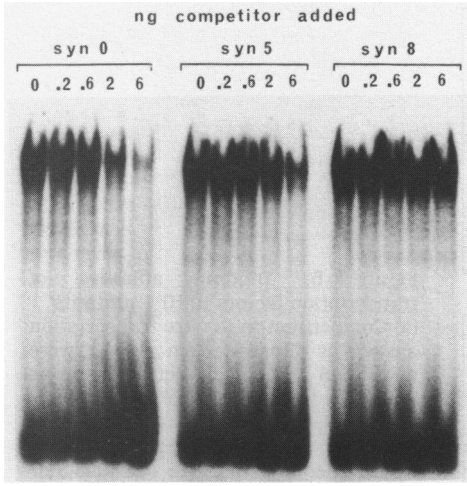

Figure 4: Competition of complex formation with different HP1 oligonucleotides as revealed by gel retardation assay. Labelled syn 0 oligonucleotide was incubated with rat liver nuclear extract and the complexes formed were analyzed by gel electrophoresis as previously described (8). Unlabelled oligonucleotides (see Fig. 2a) were added for competition as indicated.

not shown). These competition experiments were quantified by scintillation counting. The results of such an analysis are given in Figure 5b: syn 5 and syn 8 are much less efficient in competing for the complex than the wildtype sequence, whereas syn 3 and syn 15 are as efficient as the wildtype oligonucleotide syn 0. Comparing these results with the competition data we obtained from the in vitro transcription assay (Fig. 5a), we note identical competition properties of the various oligonucleotides in both assays. This strongly suggests that the complexes found in both experimental systems are identical. The 5-10 fold lower amount of oligonucleotide required for competition in the gel retardation assay reflects the seven-fold difference in the amount of nuclear extract added to the two reactions (compare Figures 5a and 5b). Taken together, these data provide strong evidence that the complex found in gel retardation strictly correlates to the functional complex leading to transcriptional activation via HP1.

Cooperation between HP1 and different TATA box sequences

Sequence comparison of the promoter region of albumin and AFP genes from Xenopus, chicken, rodents, and human revealed at least two blocks of homology (7). One block represents the HP1, the other the TATA box region at around -30. To verify that the TATA box itself is an essential compound for efficient in vitro transcription we generated a mutant in which we replaced the original two T residues of the TATA box by two G residues (Fig. 6a, construct 68GG). Such mutants have been shown to reduce the activity of the TATA box by at least 5 fold in transfection studies (12, 13). In vitro transcription with this construct gave essentially no signal of the correct size, whereas the construct PL-TG lacking HP1 but retaining the wildtype TATA box gives a weak signal (Fig. 6b and quantification in Fig. 6a). The complete absence of any transcript using 68GG as template, cannot be explained by an

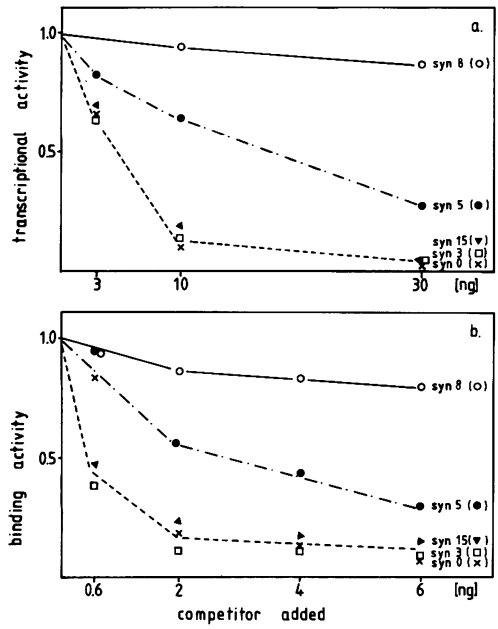

Figure 5: Binding affinities of transcription factors to HP1 mutants
(a) Competition in in vitro transcription assays as illustrated in Fig. 3 were quantified by densitometric scanning using the internal control as reference. Oligonucleotides used as competitors are indicated. Unrelated oligonucleotides (syn Δ, ERE, see also ref. 7) do not compete when tested in amounts of up to 200 ng (not shown).
b) Competition by the indicated oligonucleotides was quantified from gel retardation assays as given in Fig. 4. See text for further details.

altered initiation site that escapes detection due to the assay system used. An initiation site upstream of the -G-cassette would be unusually close to the TATA box as the G-free region starts 15 nucleotides downstream of the TATA box (7).

Comparing the TATA boxes in the promoters of the various albumin and AFP genes a distinct difference becomes obvious: The albumin genes of mouse, rat, and man contain the sequence TATATTA whereas the AFP genes contain the variant TATAAAA (see 7). Since the Xenopus albumin gene has the mammalian AFP gene sequence TATAAAA, we examined whether the two different TATA box sequences can functionally replace each other. We therefore synthesized an oligonucleotide (Bgl II - Xho I fragment, Fig. 6a) representing the TATA box region of the Xenopus albumin gene containing the corresponding point mutations. This mutated element (68 mt, Fig. 6a) was used to replace the original sequence in syn 0-TG (68 wt, Fig. 6a). In vitro transcription in a rat liver nuclear extract resulted in comparable high activity of the mutated and the wildtype sequence (Fig. 6b). Quantification of the data using two independent template preparations revealed a two-fold reduction in transcriptional activity of the mammalian albumin TATA box construct (68 mt) versus the Xenopus sequence (68 wt, Fig. 6a). This extent of transcription is still about 10 fold higher than the one obtained with a construct lacking HP1 (lane PL in Fig. 6b).

Since syn 0-TG contains HP1 and the TATA box in its natural context as found in the Xenopus albumin gene (14), we reasoned that the sequence between HP1 and the TATA box

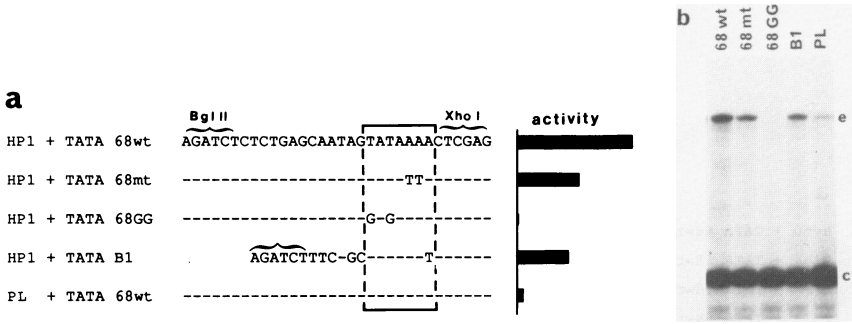

Figure 6: Mutational analysis of the TATA box region
(a) The sequence of the TATA box region as present in syn 0-TG (7) is given at the top. The Bgl II/XhoI fragment contains the Xenopus 68 kd albumin gene promoter sequence from -51 to -26 and is designated TATA 68wt. The various mutant constructs synthesized on a Pharmacia Gene Assembler are given. Identical nucleotides are indicated by a dash. The transcriptional activity of the various constructs is shown on the right and is based on the average of 2 independent in vitro transcription reactions in a rat liver nuclear extract as illustrated in (b).

might contain a third functional element we had not yet recognized. To rule out this possibility we synthesized a different TATA box region (Fig. 6a) comprising sequences from -42 to -23 of the Xenopus vitellogenin B1 gene (15). This oligonucleotide represents a third variant of a TATA box (TATAAAT), in that it has only a single base change compared to the Xenopus albumin TATA box (TATAAAA). Most importantly, the sequence between HP1 and the TATA box in this construct is quite different from that found in syn 0-TG. Again, high in vitro transcriptional activity is retained (Fig. 6b), just two-fold reduced when compared to the original construct (Fig. 6a).

These data imply that HP1 and a TATA box are sufficient to form an efficient promoter with liver-specific activity in an in vitro transcription system. This finding contrasts to the report of Gorski et al. (1) who found only weak residual activity in constructs containing the proximal promoter region (-64 to +22) of the mouse albumin gene, including the region homologous to HP1 (-64 to -52). To address this discrepancy we synthesized HP1 as found in the mouse gene and linked it to the TATA box region of the Xenopus albumin gene (Fig. 7a). In vitro transcription revealed that this construct is an efficient template (Fig. 7b) and increases the activity about 15-fold compared to the construct lacking HP1 (PL). The activity is reproducibly two-fold lower than the one found for syn 0-TG which contains HP1 as present in the Xenopus albumin gene (Fig. 7a). We also synthesized the HP1 of the mouse AFP gene (Fig. 7a) and inserted this element into the transcription vector. In vitro transcription revealed that HP1 derived from the mouse AFP gene functions in the in vitro transcription system (Fig. 7b) although it is about four-fold less efficient than the Xenopus HP1, syn 0 (Fig. 7a). Previous

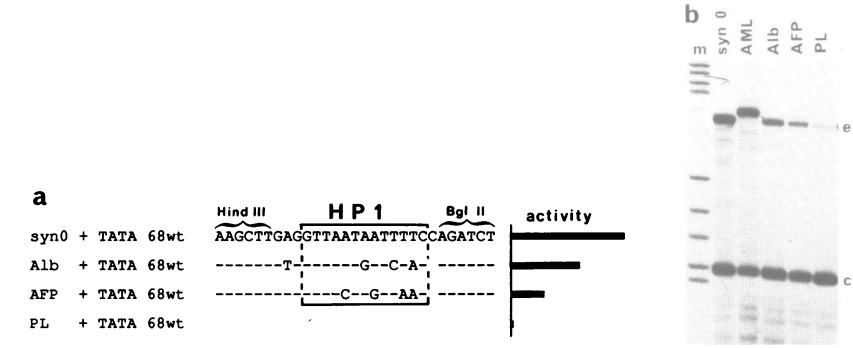

Figure 7: HP1 as found in the mouse albumin and AFP promoter is a functional element.
(a) The sequence of HP1 present in the Xenopus 68 kd albumin gene (syn 0) is given at the top. The corresponding sequences of the mouse albumin (Alb) and AFP genes (AFP) as published by Scott and Tilghman (33) were synthesized as an oligonucleotide with Hind III and Bgl II overhangs as shown. Identical nucleotides are marked by a dash. The quantified activity found for the various constructs is shown using a rat liver nuclear extract (average of 2 independent experiments). One of these experiments is included in (b).

experiments have shown that the HP1 oligonucleotides designed according to the mouse albumin or AFP genes can efficiently compete the activity of HP1 as found in the Xenopus albumin gene (8). All this provides clear evidence that the HP1 sequences as found in the mouse genes are sufficient to generate an efficient promoter for in vitro transcription using rat liver nuclear extract.

Using the region from -42 to -23 of the vitellogenin B1 gene as TATA box, we moved HP1 7 nucleotides closer to the TATA box without seeing a remarkable change in activity. To further analyze how the spacing between these two regulatory elements interferes with their function, we inserted small DNA fragments between HP1 and the TATA box using the unique Bgl II site. In vitro transcription activity did not alter significantly upon insertion of up to 46 nucleotides, but gradually decreased with larger inserts to reach background levels with a fragment of 207 bp (Fig. 8). Since we introduced fragments that do change the stereospecific alignment of HP1 and the TATA box without detecting a significant effect on transcriptional activity, we conclude that no stereospecific alignment is needed.

Based on all these data we postulate that the TATA box is absolutely essential for HP1 function in our in vitro transcription assay. Furthermore, different TATA boxes can functionally replace each other in our assay. We feel confident that HP1 and the TATA box are necessary and sufficient to form a minimal promoter conferring liver cell specific transcription in vitro.

DISCUSSION

Our data establish that two DNA elements are necessary and sufficient to constitute a promoter which is selectively active in extracts derived from liver cells. One element

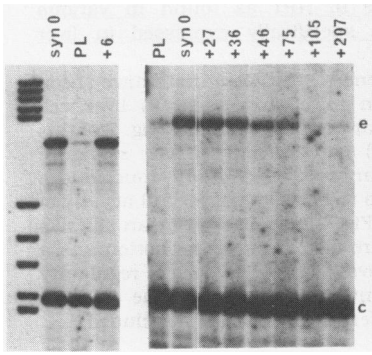

Figure 8: HP1 and the TATA box form a liver-specific promoter as long as they are in close proximity
The vector syn 0-TG containing HP1 and the TATA box was cut with Bgl II and various Sau 3A fragments derived from pBR 322 were inserted, thus moving HP1 away from the TATA box. The insert size was determined by sequencing (34). In vitro transcription was performed in a rat liver nuclear extract as illustrated in Fig. 1. The constructs are designated according to the insert size. The construct +6 was derived by insertion of syn 0 (Fig. 2) into the Hind III/Bam HI sites of PL-TG. (7).

represents the TATA box which is a ubiquitous promoter element active in many, if not all cell types. The second element is the hepatocyte-specific promoter element HP1, whose activity is restricted to hepatocytes. Our finding that a TATA box and HP1 are necessary to constitute a promoter with liver-specific activity in vitro, does not exclude the possibility that other liver-specific cis-acting elements might be equally sufficient.

The significance of the TATA box

The importance of the TATA box for accurate transcription initiation has been well documented in in vivo systems (16). In most studies the entire region containing the TATA box has been deleted to prove its functional role. In in vitro transcription systems that retain tissue-specific expression much less is known and only the effect of the deletion of the entire region has been analyzed (1, 5). In our experiments we mutated the two T residues into two G nucleotides. Such a mutation reduces the in vitro transcription at least 40-fold (Fig. 6). This effect is much more pronounced than in transfection experiments, where with similar mutants a five-fold reduction has been reported (12, 13). Changes in the TATA box sequence at positions that are not strictly conserved in natural sequences reveals that different TATA boxes mediate the liver-specific in vitro transcription. The slight differences observed may reflect the binding of proteins with different properties to the various TATA boxes. Such a heterogeneity of TATA box proteins has recently been postulated (17). Alternatively, various TATA boxes may bind the same protein, but in a somewhat different manner. In an attempt to clarify this point, we added increasing amounts of TATA box oligonucleotides as competitor to the in vitro transcription. However, competition could only be observed at very high concentrations (500 ng) and such high levels of oligonucleotides of any sequence interfer with the in vitro transcription (data not shown). The inability to compete the TATA box protein specifically may reflect its high abundance and/or its relative low binding affinity.

Our experiments show that different TATA box sequences can modulate the activity of a HP1 containing promoter. This may add another level of regulatory capacity to liver-specific gene control.

Xenopus albumin 68kd	-65	GTTAATAATTTTC	-53
mouse albumin	-64	GTTAATGATCTAC	-52
mouse α-fetoprotein	-62	GTTACTAGTTAAC	-50
rat ß-fibrinogen	-83	GTTAATATTTGAC	-95
human α_1-antitrypsin	-75	GTTAATATTCACC	-63
human transferrin	-67	GGTCAAAGATTGC	-55
		GNTNNTNNNNNNC	

Figure 9: HP1 as found in various genes specifically expressed in liver cells.
Sequences of HP1 that have been proven to be functional in liver cell nuclear extract (ref. 8 and Fig. 7 of this paper) are aligned for sequence comparison. The four nucleotides shown to be essential for full activity of HP1 (Fig. 2) are marked by an asteriks and are also given at the bottom. As a negative example the regulatory element as found in the human transferrin gene (8, 18) is included.

HP1, the promoter element conferring liver-specific expression

Mutational analysis of HP1 has shown that at least 4 positions in the 13 bp element, i.e. GNTNNTNNNNNNC, are essential for its function in the in vitro transcription system (Figure 2d). This finding agrees perfectly with our previous data on the presence of HP1 in several liver-specifically expressed genes of various vertebrate species (8): Indeed, the genes coding for the Xenopus and mouse albumin, the mouse AFP, the rat ß-fibrinogen as well as the human α_1-antitrypsin conform to the sequence GNTNNTNNNNNNC (see Fig. 9) and all these sequences have been shown to be functional HP1 sequences. On the other hand, a regulatory element that mediates liver-specific expression of the human transferrin gene (18) but differs from HP1 (8), lacks the essential nucleotide corresponding to the HP1 mutant syn 8 (Fig.9). This difference explains why the element of the transferrin promoter is distinct from HP1. Therefore the regulatory element in the transferrin gene is an example of a cis-acting liver-specific element distinct from HP1.

The sequence of HP1 as found in the Xenopus albumin gene is not a perfect palindrome. This is supported by our finding that the palindromic mutants syn 5 and syn 13 behave different (Fig. 5d): The 3rd position of HP1 (syn 5) is critical for function, whereas the 11th position (syn 13) can be changed without destroying the activity.

We have previously analyzed the same point mutations in vivo upon transfection into murine hepatoma BW1J cells (7). These data have revealed some distinct differences compared to the in vitro transcription results. Most notably, syn 8 does not inactivate HP1 in the mouse hepatoma cell line BW1J. This difference is due to the cell line used, since syn 8 is not active when transfected into HepG2 cells (data not shown).

The interaction between HP1 and the TATA box

In the case of the ß-fibrinogen gene, the sequence of HP1 is reversed (Fig. 9). This implies that HP1 can operate in either orientation. Further flexibility between the interaction of HP1 and the TATA box is inferred from the fact that natural HP1 elements are not at precisely defined positions of the promoter: The 3' border of HP1 is at -50 to -83 upstream of the cap site (Fig.

9). Such a flexibility in spacing is also observed if the distance between HP1 and the TATA box is changed (Fig. 8): As long as HP1 is within some 70 bp of the TATA box (20 bp natural distance plus 46 bp insert) it can mediate the liver-specific activation. This is reminiscent to the synergistic interaction of estrogen responsive elements (EREs) where close proximity of two elements is essential (19). Our data imply that no stereospecific alignment is required for the interaction between HP1 and the TATA box in the in vitro transcription. This extends previous experiments in which various promoter constructs have been analyzed in vivo by DNA transfection of cell cultures. Several reports have shown, that the interaction of promoter elements is independent of any specific alignment (20, 21, 22). In other situations, e.g. certain elements in the SV 40 promoter (23) or the interaction of the heat shock response element with the TATA box (24) accurate stereospecific alignment is essential for full activation potential. The basis for this difference must be due to differences in molecular mechanisms of activation. The availability of an in vitro transcription system may facilitate the analysis to resolve this problem.

The factors recognizing HP1

In an attempt to clarify why single point mutations within HP1 destroy the function of the regulatory element, we realized that the transcription factors bind the two mutants syn 5 and syn 8 with reduced affinity. This finding is based on the functional test in the in vitro transcription using mutated oligonucleotides as competitors (Fig. 3). We speculate that this reduced binding affinity is responsible for the inactivation of HP1 in these mutants. Identical changes in the binding properties are also seen in the competition experiments using the gel retardation assay. Therefore it is most likely that the complex formed in the gel retardation assay is identical to the functional transcription complex.

Identical competition properties as observed in in vitro transcription and binding assay are also documented for the mutants syn 3 and syn 15. We deduce from both experimental approaches that the factors are recognizing these mutated HP1 sequences in a similar manner as the wildtype sequence (Figure 5). Nevertheless, when inserted into the vector syn 0-TG to replace syn 0, both these mutants (syn 3, syn 15) mediate reduced transcriptional activity (Fig. 2). This reduction cannot be explained by an altered binding affinity to the regulatory element. This might implie that binding of the transcription factors per se is not sufficient for transcriptional activation. Such a surprising finding has recently also been reported for the thyroid hormone receptor which can bind to the ERE (estrogen responsive element) with similar affinity as to the TRE (thyroid responsive element) without exerting any stimulatory effect (25). However, there are alternative interpretations, e.g. that syn 3 and syn 15 have acquired the capacity to bind other proteins. Final proof will require a transcription system consisting of known purified components.

Recent experiments in other laboratories have also revealed the liver-specific promoter element HP1 and the corresponding binding factors (HNF1 in ref. 9; LFB1 in ref. 5, 26; APF in

ref. 27). However, in these cases an extensive mutational analysis of the function mediated by the regulatory element is not available. It is not clear whether these binding proteins are identical or rather belong to a family of related factors.

The structure of a minimal promoter conferring liver-specific gene expression

Our results reveal that liver-specific gene expression can be mimicked in an in vitro transcription system. The template needed requires only two components, the TATA box and HP1. This apparent low complexity contrasts with the reported highly complex promoter structure of other liver-specific expressed genes (e.g. mouse/rat albumin in ref. 1, 28, 29, 30; human α_1-antitrypsin in ref. 10, 31). We believe that HP1 represents a key control element for liver-specific expression and that it has therefore been conserved during evolution between the frog and mammals. The observed inability of a mouse albumin promoter construct retaining HP1 and the TATA box region (-64 to +22) to serve as an efficient transcriptional template (1), contrasts with our results. Indeed, we have provided direct evidence that the mouse albumin HP1 is functional since this element confers liver-specific transcription if combined with the Xenopus TATA box region (Fig.7). This discrepancy might reflect the presence of a negative element between HP1 and the TATA box of the mouse promoter. This interpretation seems plausible since this region has been conserved in mammalian albumin genes (30) and differs from the Xenopus sequence (14). Furthermore, a protein binding site between HP1 and the TATA box has been reported for the mouse albumin gene (site A in ref. 28). Alternatively, the sequences downstream of the TATA box of the mouse albumin gene (-24 to +22) may be responsible for this discrepancy since the corresponding sequence is lacking in the Xenopus constructs that do not include any sequence downstream of the TATA box. Consistent with this interpretation are results obtained from transfected HepG2 cells (data not shown). In this instance, Xenopus albumin-CAT constructs containing the cap region up to position +19 are substantially less active than those just retaining the TATA box region (up to -26).

Apparently, sequences upstream of HP1 are dispensable for transcription of the Xenopus albumin gene (7). However, sequence comparison between the two Xenopus albumin genes has revealed extensive sequence homologies in the 5' flanking region implying functional importance, (14). We assume that the presumed other regulatory elements are not functional in mammalian cells. This might reflect the fact that these elements play a minor role in liver-specific expression, i.e. they reflect some modulatory units.

In conclusion our data establish that liver-specific transcription is obtained in vitro with a minimal promoter retaining two functional elements, one of which is recognized by tissue-specific factors. We propose that liver-specific gene expression depends on the presence of transcription factors specific for hepatocytes. To test this proposal, it will be most important to investigate whether the transcription of genes containing HP1 is induced in a given cell if the transcription factors recognizing HP1 are provided.

ACKNOWLEDGEMENTS

We thank R. Burkhardt for the synthesis of the oligonucleotides, M. Kleinhans for excellent technical assistance, P. Herrlich and K. Roß for critical reading of the manuscript. The work was supported by the Deutsche Forschungsgemeinschaft.

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