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The Rat Albumin Promoter: Cooperation with Upstream Elements Is Required when Binding of APF/HNF1 to the Proximal Element Is Partially Impaired by Mutation or Bacterial Methylation

FRANÇOIS TRONCHE,[1] ANNA ROLLIER,[2] INGOLF BACH,[1] MARY C. WEISS,[2] AND MOSHE YANIV[1]*

Unité des Virus Oncogènes[1] and Unité de Génétique de la Différenciation,[2] UA 1149 du Centre National de la Recherche Scientifique, Département de Biologie Moléculaire, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France

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We have characterized in the accompanying paper (P. Herbomel, A. Rollier, F. Tronche, M.-O. Ott, M. Yaniv, and M. C. Weiss, Mol. Cell. Biol. 9:4750–4758, 1989) six different elements in the albumin promoter. One of them, the proximal element (PE), is the binding site for a strictly liver specific factor, APF/HNF1. This binding site contains a bacterial DAM DNA methylase methylation target sequence which, when methylated, decreases the affinity of the protein for this element. When the different albumin promoter constructions were prepared in an *Escherichia coli* deoxyadenosine methylase-negative strain, the respective contributions of the elements to the overall promoter activity were strikingly different. An intact proximal element plus the TATA box gave almost full transcriptional activity in transient transfection experiments and only in differentiated hepatoma cells of line H4II, whereas the distal elements (distal element III [DEIII], the NF1-binding site DEII, and the E/CBP-binding site DEI) had become essentially dispensable. Mutations affecting the CCAAT box showed only a two- to threefold decrease. When PE was methylated, mutated, or replaced by the homologous element from the α-fetoprotein gene, activity in the context of the short promoter (PE plus the TATA box) was abolished. However, activity was restored in the presence of the upstream elements, showing that cooperation with factors binding to the CCAAT box and distal elements favors the functional interaction of the liver-specific APF/HNF1 factor with lower-affinity binding sites.

Developmentally regulated and tissue-specific transcription of genes probably requires a sequence of events, including modification in specific chromatin sites in particular cells, the maintenance of this determined state through cell division, and the assembly of a competent transcription initiation complex at the correct site on the DNA. Most attempts to elucidate the mechanisms underlying tissue-specific transcription involve the characterization of *cis*-acting elements after transfection of chimeric constructs into appropriate cell lines and of their interaction with specific *trans*-acting factors in cell extracts. Although these studies address only the last step in the gene activation pathway mentioned above, they have been fruitful: in a number of systems, elements endowed with tissue-specific activity have been identified in differentiated cell lines (lymphocytes [16], pituitary cells [26], and hepatocytes [13, 34; this study]), and some of the tissue-specific *trans*-acting factors have recently been cloned (Otf-2/Oct-2 [7, 25, 36] and GHF-1 and Pit1 [3, 27]). In the systems analyzed, tissue-specific transient expression has been found to result from the action of one (human growth hormone [3], prolactin [30], immunoglobulins H and K [38, 39], and α- and β-fibrinogen [10]) or two (α1-antitrypsin [28]) tissue-specific factors, mostly in combination with ubiquitous factors (α-fetoprotein [AFP] [17]).

However, several clues indicate that potentially important sequence determinants may be overlooked when this approach is used. First, transfected promoters from tissue-specific genes are sometimes active in heterologous cells (γ-fibrinogen [29] and tyrosine aminotransferase [23]). Second, in the vicinity of the *cis* elements identified by transient expression are often found high-affinity binding sites for known transcription factors to which no clear function can be ascribed in this context (NF1 in the S promoter of hepatitis virus [11] and the albumin promoter [this study]; and MLTF/USF in the γ-fibrinogen promoter [29]). Third, additional sequences sometimes prove to be required in order to obtain the correct expression pattern in transgenic mice (18). This is the case for the albumin gene (31): in transgenic mice, abundant and liver-specific albumin gene transcription requires the presence of a region located between −8.5 and −10.4 kilobases in addition to the promoter.

We have previously shown that the 150 base pairs upstream of the rat albumin gene constitutes a fully functional promoter in the differentiated hepatoma cell line H4II (32), where it is as active as the simian virus 40 early promoter (20). In striking contrast, in dedifferentiated H5 hepatoma cells (12) or in fibroblasts, this promoter is 100-fold less active. The homologous region of the mouse albumin gene was shown to be required for tissue-specific transcription in vitro, using nuclear extracts from liver or other organs (15). We have shown that the tissue-specific promoter activity obtained after transfection relies on six independent positive elements, four of which bind a characterized *trans*-acting factor in vitro. Distal element II (DEII), at nucleotide −120, binds NF1/CTF, DEI at −90 binds C/EBP20 (1, 5, 27), a CCAAT box at −83 binds ACF/NFY (33), and a proximal element (PE) at −60 binds the APF factor (4), also designated HNF-1, LF-B1, or HP1 in other contexts. Present only in hepatocytes and differentiated hepatoma cells, this last factor also participates in the transcriptional control of several other liver-specific genes, including α1-antitrypsin (28), pyruvate kinase (S. Vaulont, personal communication), AFP (14, 17), and transthyretin (8) genes.

The strict tissue distribution of the APF/HNF1 factor, combined with the presence of its target sequence in a

* Corresponding author.

number of liver-specific genes, implies that the APF-PE interaction may be critical in hepatocyte-specific transcription. Since the albumin promoter PE includes a bacterial deoxyadenosine methylase (DAM) methylation site, we have prepared plasmid DNA in an *Escherichia coli dam* mutant strain to investigate the possible effect of this modification on promoter activity. We show here that in the absence of bacterial methylation in PE, the sequence requirements for efficient and tissue-specific transient expression are drastically different: among the elements previously defined, only the TATA box and PE are now required for transcriptional activity. In fact, we show that more generally, if the affinity of PE for APF is decreased, even only moderately, by methylation or by mutation, maintenance of promoter activity absolutely requires cooperation of the four more upstream elements.

MATERIALS AND METHODS

Culture conditions, DNA transfections, and enzyme assays. Rat hepatoma H4II cells and the dedifferentiated derivative H5 cells were grown and transfected, and enzymatic assays were performed, as previously described (20).

Plasmid preparation. Large-scale preparations of the different constructions were obtained from *E. coli* K-12 DH5-1-transformed bacteria. A *dam* mutant strain (GM113 dam3) was used for the preparation of plasmid DNA unmethylated at DAM methylation sites (5′-GATC-3′) (19). We have controlled for the absence of DAM methylation by restriction analysis with the *Mbo*I enzyme, which recognizes GATC and cleaves only unmethylated DNA.

Plasmid construction. The construction of linker-scanning (LS) and 5′ deletion mutants (−103, −93, and −54) is described by Heard et al. (20) and Herbomel et al. (21). We chose another strategy to obtain additional deletion or point mutants in the promoter and to prepare chimeric promoters in which the rat albumin PE was replaced by the proximal AFP homologous element. We synthesized a set of six double-stranded oligonucleotides with cohesive ends: TF1 (GATCTCGGGATTTAGTTAAACAA) and TF12 (AAAA AGTTGTTTAACTAAATCCCGA), TF2 (CTTTTTTTTT TCTTTTTGGCAAGGAT) and TF11 (ATACCATCCTTGC CAAAAAGAAAAA), TF3 (GGTATGATTTTGTAATG) and TF10 (CTACCCCATTACAAAATC), TF4 (GGGTAG GAACCAATGAAATGAAAG) and TF9 (ACTAACCTT TCATTTCATTGGTTC), TF5 (GTTAGTGTGGTTAATGA TCTACAGTTA) and TF8 (AACCAATAACTGTAGAT CATTAACCAC), and TF6 (TTGGTTAGAGAAGTATATT AGAGCGAGTTTCTCTGCACACAGACCA) and TF7 (AG CTTGGTCTGTGTGCAGAGAAACTCGCTCTAATATACT TCTCT).

After hybridization and ligation, a wild-type promoter extending from positions −151 to +4 was reconstituted between the *Bgl*II and *Hin*dIII restriction sites of the chloramphenicol acetyltransferase (CAT) expression vector. The desired point mutations or substitutions were introduced by replacing the relevant standard oligonucleotide with the desired variant (see Fig. 4). By this approach, it was possible to generate specifically and with equal facility any simple or complex mutation in the albumin promoter. Intact or mutant short promoters (−68/+4) were constructed with the same approach, using PE (TF5 and TF8) and TATA (TF6 and TF7) oligonucleotides plus a *Bgl*II-*Pst*I adaptor: 5′-GATC TCTGCA-3′ and 5′-ACTAACTGCAGA-3′.

Oligonucleotides were synthesized on a Pharmacia Gene Assembler, purified in 18% urea denaturing polyacrylamide gels, and phosphorylated (24). Annealing was performed in kination buffer containing 4 μM each required oligonucleotide, and then 100 ng of vector pUMS-SB1 (linearized by *Bgl*II-*Hin*dIII digestion) was added for ligation. Each construction was controlled by supercoil sequencing according to the method of Chen and Seeburg (6).

Cross-competition in gel mobility shift assay. A 600-ng sample of the oligonucleotide PE56 (see Fig. 3) (4) was 5′ end labeled and split into two parts; 300 ng was methylated in the presence of 75 U of DAM (New England BioLabs, Inc.), and 300 ng of unlabeled PE56 was methylated in parallel under the same conditions. Methylation efficiencies were tested on equal samples by *Mbo*I digestion and polyacrylamide gel migration (after 5′ end labeling of a sample of the unlabeled PE56).

The mobility shift assays were done as described by Cereghini et al. (4), using 4 μg of rat hepatocyte nuclear protein extracts and 0.6 ng of labeled methylated and unmethylated PE56 in the presence of different amounts of nonradioactive competitor oligonucleotides. The bands corresponding to the APF-PE56 complex were cut from the dried gel, and radioactivity was measured in a scintillation counter.

RESULTS

In another study (21), we localized six distinct positive elements in the albumin promoter, DEIII, DEII, DEI, CCAAT box, PE, and TATA box, all of which are required for efficient and tissue-specific transient expression. Only one (PE) appeared to bind a clearly liver specific factor in vitro (APF/HNF1 [4]). At this point, we noticed that this element contains an *Mbo*I restriction site (−53 GATC −50), where the adenine on each strand is methylated by DAM (19) in the standard *E. coli* K-12 strains used for the preparation of plasmids. At least one of the two methylated adenines (A/−51) on the coding strand has been shown by methylation interference to be a major contact point for APF in vitro (4). No other DAM or deoxycytosine methylase (DCM) methylation site is present in the rat albumin promoter. To test whether DAM methylation of the APF-binding site could interfere with promoter activity, we prepared a number of constructions in an *E. coli dam* mutant strain and tested their expression after transfection of differentiated (H4II) or dedifferentiated (H5) rat hepatoma cells.

DAM methylation of PE does not change the transcriptional activity of the −151 promoter. We first tested in H4II cells the complete (−151/+16) promoter previously described as well as a synthetic (−151/+4) promoter (see Materials and Methods) that had similar activities when prepared in dam^+ bacteria (dam^+ DNA). When these two plasmids were prepared in *dam* mutant bacteria, the CAT activity obtained in H4II cells was 140% of that obtained with plasmid DNA produced in dam^+ bacteria. This finding shows that bacterial DAM methylation (in the promoter or at other sites in the plasmids) does not significantly alter the efficiency of transcription from the complete Δ−151 promoter.

PE and the TATA box are sufficient for efficient transcription of *dam* mutant unmethylated DNA in H4II cells. When we tested progressive 5′ deletion mutants of this promoter, the results obtained with dam^+ and *dam* mutant DNA were strikingly different (Fig. 1 and 2). With dam^+ DNA, we had previously shown that deletion of the three most distal elements (DEI, -II, and -III) led to a cumulative, more than 100-fold decrease in transcriptional activity. When *dam* mutant DNA was used, however, all sequences upstream of

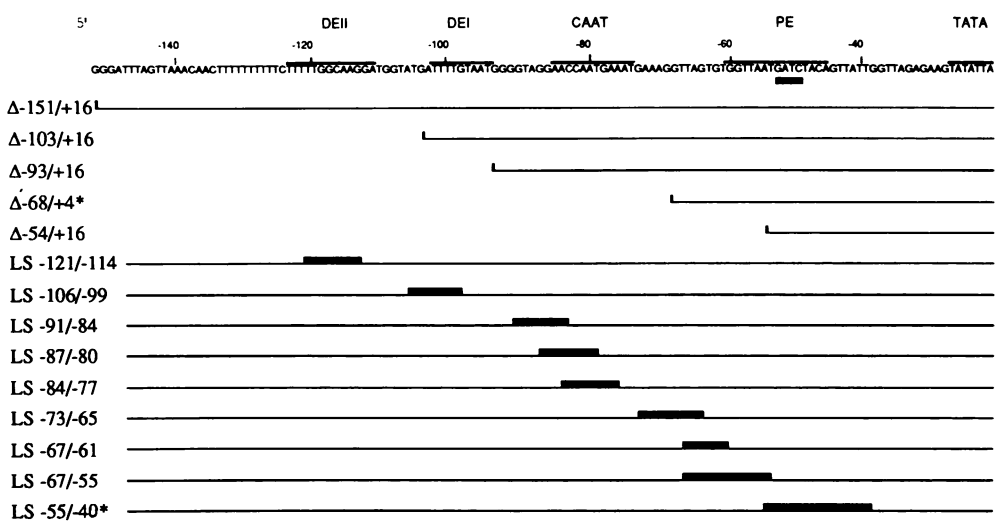

FIG. 1. Activity of rat albumin promoter mutants (deletion and LS) in albumin-positive H4II and albumin-negative H5 hepatoma cells and the effect of DAM methylation in PE on this activity. Except for two plasmids (*), the results of *dam*$^+$ mutants were taken from references 20 and 21. Black boxes represent the location of a given LS mutant relative to the rat albumin promoter sequence indicated above. The CAT activity obtained after transient expression was normalized for β-galactosidase activity obtained in the same extract and expressed as percentage of the activity of the corresponding *dam*$^+$ or *dam* mutant (i.e., −151/+16 or −151/+4) intact rat albumin promoter. The lines above the sequence indicate highly conserved regions of this promoter. The DAM methylation site is underlined.

the proximal element became essentially dispensable. Only deletion of the CCAAT element (*dam* mutant Δ−68) caused a twofold reduction in activity. Further deletion (*dam* mutant Δ−54) into the proximal element (APF-binding site) abolished promoter activity. Thus, in this context, only 68 base pairs, including the TATA box and APF-binding site, provided high and tissue-specific promoter activity (see below).

Phenotype of mutants affecting the conserved DEII, DEI, or CCAAT element. We next examined the effect of DAM methylation on the activity of LS mutants that destroy separately each of the five elements (Fig. 1). Whereas the *dam* LS mutant (LS −106/−99) replacing DEI roughly equalled the *dam* mutant Δ−151 promoter, a mutation (LS −121/−114) substituting the NF-1 binding site (DEII) caused a twofold increase in transcriptional activity. More intriguing were the results obtained with the mutants that modify the CCAAT box. Whereas LS −84/−77, which totally replaces the CCAAT consensus sequence, had no effect, replacement of the nucleotides located just upstream caused a twofold increase in CAT activity (LS −91/−84). However, a mutant (LS −87/−80) partially overlapping these two mutants induced a three- to fourfold decrease in promoter activity (Fig. 1).

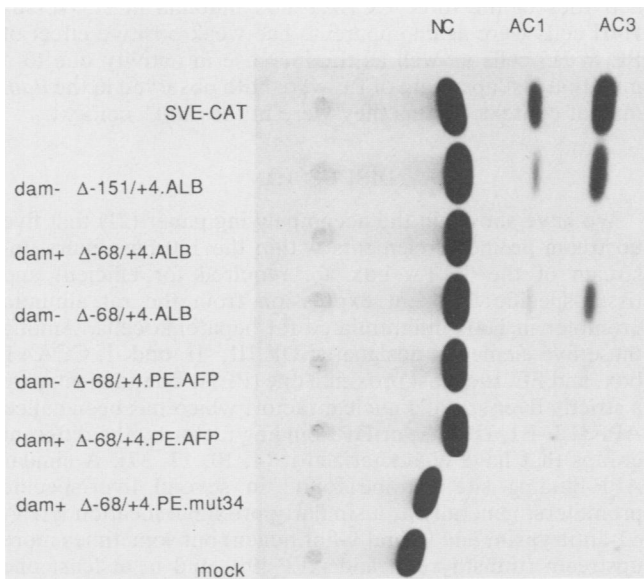

FIG. 2. CAT activities obtained with some of the albumin promoter construction shown in Fig. 4 after transfection and expression in H4II cells. NC, Native chloramphenicol; AC1, 1-acetyl-chloramphenicol; AC3, 3-acetyl-chloramphenicol.

PE is the only element important for transient expression in differentiated H4II hepatoma cells. Among all of the LS mutants tested as *dam* mutant DNA, the only ones showing a strong reduction in activity were those in which PE had been altered. LS −55/−40 destroys the downstream moiety of the APF-binding site, including the GATC DAM methylation site. Its activity was equally low whether *dam*$^+$ or *dam* mutant DNA was used. This finding confirms that the effects of DAM methylation concern the binding of APF to the proximal element. In addition, this result excludes any influence of the methylation of surrounding plasmid sequences on the level of transcription from the rat albumin promoter region. Similarly, LS −67/−55, which alters the upstream moiety of the APF-binding site while leaving the *Mbo*I site intact, displayed a very low activity both as *dam*$^+$ and as *dam* mutant DNA.

Finally, a mutation (LS −67/−61) adjacent to the upstream border of the APF-binding site, which was as deleterious as deletion of the APF site itself in the *dam*$^+$ context, caused only a twofold decrease in the *dam* mutant context. Therefore, nucleotides −67 to −61 are apparently required for the rescue by more distal elements of the deleterious effect of alterations within the proximal element. A mutation in this region could either inhibit the binding of a factor that stabilizes APF/HNF1 binding to the proximal region or

block the cooperation of the upstream elements with the proximal element, as argued below.

Thus, the cooperation of the more upstream elements is required only when the APF-binding site is methylated. Is this phenomenon a reflection of a lower affinity of APF for the proximal element when it is methylated?

Methylation of adenines −51 and −52 affects the binding of APF/HNF1. We have defined by gel shift assays a double-stranded oligonucleotide (PE 56 [−63/−41]) encompassing the PE element that specifically interacts with APF/HNF1 present in liver or differentiated hepatoma cells (4). To examine whether methylation of adenines −51 and −52 affects the affinity of APF/HNF1 present in rat liver nuclear extracts for its target sequence, we carried out similar gel retardation experiments, using either the DAM-methylated or unmethylated PE56 as probe, in the presence of increasing amounts of methylated or unmethylated cold PE oligonucleotides (Fig. 3).

The superposition of the competition curves obtained with methylated and unmethylated labeled probe competed with the homologous oligonucleotides (i.e., methylated and unmethylated, respectively) indicated that the correct amounts of oligonucleotides were used. The reciprocity between curves obtained with cross-competitions allowed us to conclude that methylation in position N6 of adenines −51 and −52 caused about a fourfold decrease in the affinity of APF/HNF1 for the albumin PE in vitro. Similar results were obtained with nuclear extracts derived from H4II cells (S. Cereghini, personal communication). It is worth noting that DAM methylates adenines −51 and −52 to 6-aminomethyl purine, with the methyl group localized in the major groove of the double helix, and that methylation by dimethyl sulfate in the N3 position of the A −51 residue in the minor groove interferes with the binding of APF/HNF1 (4). Thus, APF appears to interact with both sides of the double helix.

The effects of PE DAM methylation suggest that a requirement for the cooperation of the more upstream promoter elements is linked to a decreased affinity for APF/HNF1. If this is so, a similar effect should also be obtained with point mutants in PE that affect this affinity in the same way as DAM methylation.

Mutations in PE are complemented by the cooperation of the upstream elements. The bases to mutagenize were chosen on the basis of dimethyl sulfate interference contacts (4) and of sequence conservation of this element among liver-specific promoters. Two combinations of double point mutations (PE.mut34 and PE.mut56; Fig. 2 and 4) abolished the activity of the *dam* mutant Δ−68 promoter in H4II cells. However, in the presence of the four upstream elements, DEIII, DEII, DEI, and CCAAT, they caused only a three- to fourfold decrease in transcriptional activity.

The rat AFP promoter contains a similarly located APF/HNF1-binding site (with no DAM methylation site) that also appears to be crucial for tissue-specific promoter activity. Since the affinity of APF/HNF1 for this site has been reported to be lower than for the rat albumin proximal element (4), we replaced the latter by the former within the albumin promoter. In the presence of the more upstream elements, this construct was as efficient as the natural albumin promoter. However, deletion of the sequences upstream of PE abolished transcription (Fig. 2 (*dam* mutant and *dam*$^+$ Δ−68/+4.PE.AFP) and 4 (Δ−68/+4.PE.AFP and Δ−151/+4.PE.AFP). Thus, within the albumin promoter, the APF/HNF1-binding site from the AFP promoter behaves just as the DAM-methylated albumin APF/HNF1 site.

To further investigate the dependence of the AFP or mutated albumin PE on the cooperation of the more upstream elements, we combined these mutations with point mutations either in DEII or in the CCAAT box. Such single point mutations had almost no effect when PE was methylated or replaced by the AFP homolog, but they abolished transcription when combined with two double PE mutations that caused only a three- to fourfold decrease in activity with intact upstream sequences (Fig. 4).

Upstream elements (CCAAT and DEI) are required for low transcriptional activity in H5 cells. H5 cells, derived from H4II, fail to express the entire group of liver-specific genes, including the albumin gene. However, a low level (about 1% of simian virus 40 early promoter activity) of faithful initiation takes place at the albumin promoter in the transient expression assay. When *dam* mutant DNA is used, a two- to threefold higher level is obtained. This result may reflect an effect of methylated DAM sites in the expression vector that was undetectable in H4II cells, in which the promoter activity is 100–200 fold higher.

We have shown in the accompanying paper (21) that in a *dam*$^+$ context, the very low promoter activity observed in H5 cells depends first on the CCAAT box, then on DEI, and only marginally on PE. The latter point correlates with the lack of detectable APF/HNF1 in H5 cells, in which a different but related protein (vAPF) interacts with PE (4). Since DAM methylation similarly affects APF/HNF1 and vAPF-binding affinities (S. Cereghini, personal communication), we wanted to see whether the methylated or unmethylated state of PE would change the relative contributions of the various promoter elements in H5 cells. To do so, most of the mutants described above were transfected into H5 cells both as *dam*$^+$ and as *dam* mutant DNAs. In contrast to the results for H4II cells, the DAM methylation status of PE did not significantly influence the activity of the various deletion and substitution mutants in H5 cells (Fig. 1 and 4).

Finally, since vAPF binding is similarly affected by bacterial methylation, the lack of a difference in activity between *dam*$^+$ and *dam* mutant DNAs in H5 cells confirms that vAPF is not an active transcription factor in these cells. With *dam* mutant DNA, the differences in the relative activities of the three CCAAT box mutants in H5 versus H4II cells were again apparent. The weak positive effect of PE in H5 cells as well as the increase in activity due to a mutation just upstream of PE were both observed in the *dam* mutant context, just as they were in the *dam*$^+$ context.

DISCUSSION

We have shown in the accompanying paper (21) that five upstream promoter elements within the 120 base pairs upstream of the TATA box are required for efficient and tissue-specific transient expression from the rat albumin promoter in H4II differentiated rat hepatoma cells. Among these five elements, designated DE III, -II, and -I, CCAAT box, and PE, the most proximal one (PE) is bound in vitro by a strictly liver specific nuclear factor, which has been called APF, LF-B1, HNF-1, or HP1 binding factor by the different groups that have characterized it (4, 10, 13, 37). A similar APF-binding site can be found in several liver-specific promoters, generally at a similarly proximal location (AFP, α1-antitrypsin, and β- and γ-fibrinogen) but sometimes more upstream (transthyretin and AFP [9]), and in at least one liver-specific far upstream enhancer (human AFP [35]). In at least four of these genes, the proximal APF/HNF1-binding site has been shown to be crucial for tissue-specific transient expression in transfected hepatoma cells. Thus, APF/HNF1

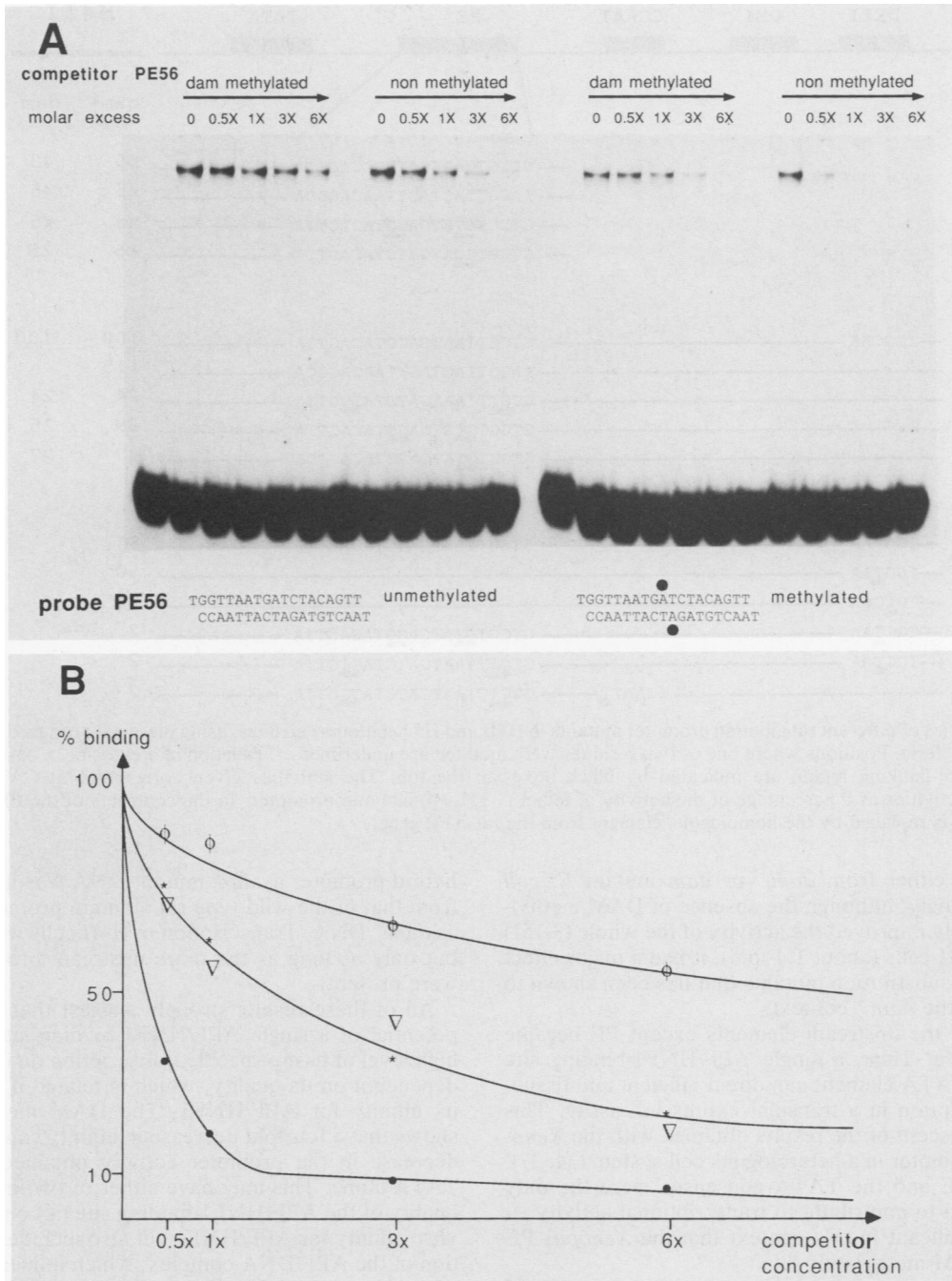

FIG. 3. Effect of DAM methylation in PE (A −51 and −52) on the formation of the APF-PE complex with liver nuclear extracts. (A) Gel electrophoretic mobility shift assays were carried out as described in Materials and Methods. Complexes obtained with 4 μg of nuclear extracts and 0.6 ng of DAM-methylated or unmethylated PE56 oligonucleotide were competed with the indicated amounts of nonradioactive, methylated or unmethylated PE56. Adenines methylated in N6 by DAM are marked (●). (B) The amount of bound oligonucleotide was quantitated by cutting the bands corresponding to the APF/HNF1-PE56 complex and measuring the radioactivity in a scintillation counter. The fold excess of competitor oligonucleotide is indicated on the abscissa. The amount of residual complex, expressed as a percentage of that found in the absence of competitor (100%), is indicated on the ordinate. For experiments performed with unmethylated probe, Φ and Δ correspond to methylated and unmethylated competitor, respectively. With the DAM-methylated probe, the curves obtained with unmethylated competitor (●) and with methylated competitor (*) are indicated.

is probably a key factor in the activation of many liver-specific functions.

Unlike the case with other genes of this class, the APF/HNF1-binding site of the mouse and rat albumin promoters contains a DAM methylation site (GATC), so that the adenines on each strand of this sequence are methylated at position N6 when the plasmid DNA is prepared from the usual *E. coli* K-12 strains. To determine whether this methylation could interfere with promoter activity, we tested the behavior of many albumin promoter mutants transfected as

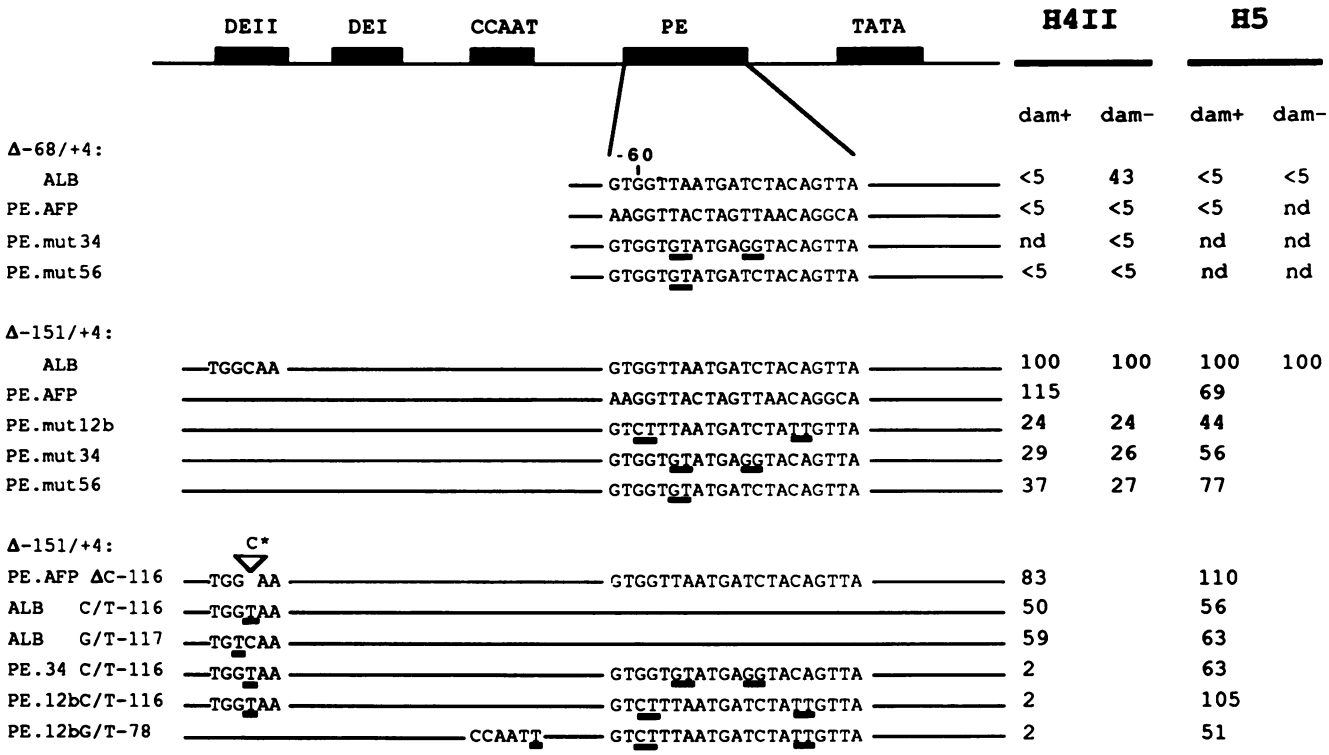

FIG. 4. Activities of different rat albumin promoter mutants in H4II and H5 hepatoma cell lines, using plasmids produced in normal (*dam*$^+$) or *dam* mutant bacteria. Positions where one or two residues were mutated are underlined. *, Deletion of a cytosine. Conserved elements in the rat albumin 5'-flanking region are indicated by black boxes at the top. The activities given correspond to CAT normalized for β-galactosidase activities as a percentage of the activity of intact (−151/+4) albumin promoter. In the contructs of the PE-AFP series, the proximal element is replaced by the homologous element from the rat AFP gene.

DNA prepared either from *dam*$^+$ or *dam* mutant *E. coli* strains. Surprisingly, although the absence of DAM methylation only slightly improved the activity of the whole (−151) promoter in H4II cells (about 1.4-fold), it had a major effect on deletion and substitution mutants that has been shown to be defective in the *dam*$^+$ context.

In fact, all of the upstream elements except PE became quasi-dispensable. Thus, a single APF/HNF1-binding site upstream of a TATA element can direct efficient and tissue-specific transcription in a transient expression assay. This finding is reminiscent of the results obtained with the *Xenopus* albumin promoter in a heterologous cell system (34, 37), in which the PE and the TATA consensus were the only sequences found to contribute to transcriptional activity. It is certainly significant in this context that the *Xenopus* PE displays no DAM methylation site.

Gel retardation competition assays have shown that DAM methylation of the rat albumin PE causes a fourfold decrease in the affinity of the PE for APF/HNF1 in vitro. Under these conditions, cooperation of the more upstream elements is required for efficient and tissue-specific transient expression in differentiated hepatoma cells. Interestingly, the same requirement for the cooperation of more upstream promoter elements has been found for the similarly located APF/HNF1-binding sites in the α1-antitrypsin and AFP promoters (13, 17), neither of which is methylated in *E. coli*. In a separate study (4), the affinities of these two sites for APF/HNF1 have been shown to be similar and clearly lower than that of the homologous unmethylated site in the rat albumin promoter.

When we substituted the PE within the rat albumin promoter with that of the AFP promoter, the behavior of this hybrid promoter as *dam* mutant DNA was indistinguishable from that of the wild-type rat albumin promoter transfected as *dam*$^+$ DNA. Transcription in H4II cells was very efficient but only as long as the more upstream promoter elements were present.

All of these results strongly suggest that the remarkable potential of a single APF/HNF1-binding site to promote a high level of tissue-specific transcription on its own is highly dependent on its quality, which is related if not identical to its affinity for APF/HNF1. The DAM methylation effect shows that a fourfold decrease in affinity can cause a 50-fold decrease in the promoter activity obtained from PE and TATA alone. This may have either of two meanings: (i) the quality of the APF/HNF1-binding site not only reflects its in vitro affinity for APF/HNF1 but also includes the conformation of the APF/DNA complex, which influences its interaction with the preinitiation complex, or (ii) affinity is indeed the only determinant, but the activity is a nonlinear function of the occupancy state of the site by APF/HNF1. Such a situation is predicted when two factors bound to DNA (here APF/HNF1 and the TATA sequence-binding factor, TFIID) affect transcription by binding to the same third factor (here RNA polymerase II or its general cofactors) (P. Herbomel, Ph.D. thesis, University of Paris, Paris, France, 1989).

Nature of the cooperation between the proximal and the more upstream elements. When the affinity of the proximal APF/HNF1-binding site is somewhat lower than that of the wild-type rat albumin element, more upstream elements are required to restore full transient expression.

On the basis of the critical nucleotides revealed by methylation interference data obtained in vitro, we have also constructed two multiple point mutants in PE which impede

its function more severely than DAM methylation but still less than its total deletion. These two mutants show no activity, whether their DNA is *dam*$^+$ or *dam* mutant, in the absence of the more upstream elements. In their presence, promoter activity is restored to about one-third that of the complete wild-type promoter. Interestingly, this rescue depends much more on the integrity of each of the more upstream elements than it does when the proximal element is either the wild-type methylated rat version or the rat AFP version. A single point mutation in DEII or the CCAAT element is sufficient to totally abolish the *cis* complementation, whereas similar point mutations have almost no effect when combined with the AFP or methylated wild-type albumin version of the proximal element: in the latter two cases, cooperation of the upstream elements is also essential, but drastic mutations in them are necessary to abolish it.

Finally, two observations may further suggest a direct interaction between APF/HNF1 and the more upstream bound factors. In the *dam*$^+$ context, we showed that a mutation just upstream of the APF/HNF1-binding site was as deleterious as deletion of the whole PE. However, we show here that when the transfected DNA is *dam* mutant, this mutation only mildly affects transcription efficiency. This finding may suggest that this mutation impedes the cooperation of the more upstream elements, possibly by altering the local conformation of the DNA. If this is the case, then the position of this mutation suggests that APF/HNF1 may directly interact with the factors bound upstream. In this respect, it is worth recalling that unlike that of DEI and CCAAT, the contribution of DEII and DEIII observed in the *dam*$^+$ context is tissue specific. Altogether, this may also mean that the positive action of the ubiquitous factor NF1 bound at DEII depends on its direct interaction with APF/HNF1 bound at the PE.

Possible roles of DEIII, -II, and -I and the CCAAT element in the activation of the albumin promoter. Although the PE of the endogenous albumin promoter cannot be methylated by the mammalian cell machinery, the more upstream elements are very likely to be as important for its developmental activation as they are for the other liver-specific promoters that also display a crucial APF/HNF1 proximal site. The albumin distal elements are strongly conserved from chicken to humans; DEI and the CCAAT element are among the strongest binding sites known for CBP and ACF/NF-Y, respectively (2). Furthermore, it is already known that the sequence requirements for tissue-specific activity of the albumin promoter in transient expression assays in cell culture are more relaxed than in the organism: efficient and liver-specific transcription in transgenic mice requires the cooperation of a far upstream enhancer (at −10 kilobases [31]) in addition to the albumin promoter region. Therefore, we suggest that in addition to the proximal APF/HNF1-binding site, both the more upstream elements of the promoter and the far upstream enhancer are required for proper developmental activation of the gene, at least in a transitory fashion for the opening of an initially highly condensed and methylated chromatin and formation of the correct complex of transcription factors at the promoter. Furthermore, since the ability of the PE to ensure efficient transient expression depends so strongly on the binding of APF/HNF1, the promoter upstream elements could enhance its binding under developmental or physiological conditions in which its available concentration is low.

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ADDENDUM IN PROOF

S. Lichtsteiner and U. Schibler (Cell **57**:1179–1187, 1989) have recently shown that DAM methylation of the HNF-1 binding site of the mouse albumin promoter decreases the affinity for the purified factor and affects the promoter activity in vitro. As in vivo, the effect was more pronounced with shorter promoter constructs.

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