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Cell type-specific negative regulatory element in the control region of the rat α-fetoprotein gene

(enhancer/silencer/promoter specificity)

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ABSTRACT **Albumin and α-fetoprotein are evolutionarily related genes that show differential and complex patterns of regulation during development. We investigated the role of the sequences flanking the transcription initiation site of the rat α-fetoprotein gene in transient transfection assays of hepatic and nonhepatic cell lines. Chimeric flanking regions and deletion analysis have defined the following three functionally different regions: (*i*) a cell type-specific enhancer(s), encompassing 3 kilobases, located between −7 kilobase pairs and −4 kilobase pairs; (*ii*) a cell type-specific promoter, inactive in the absence of an enhancer and comprising at most the 180 base pairs upstream from the site of transcription initiation; and (*iii*) a 550-base-pair region, located between the promoter and the enhancer (at −3.5 kilobases), down-regulates transcription initiation in a cell type-specific manner and is also capable of repressing heterologous promoters. The implications of these findings with respect to the complex pattern of AFP regulation are discussed.**

Tissue-specific phenotypes are imparted by the activation of specific sets of genes in cell lineages during development. α-Fetoprotein (AFP) and albumin are evolutionarily related serum proteins that show complex patterns of developmental and tissue-specific synthesis (1). During gestation, the AFP and the albumin genes are simultaneously activated in fetal liver. mRNAs for both genes reach high levels, then AFP transcription is shut off at birth, while albumin transcription continues in the adult at high levels. In the rat yolk sac, only AFP mRNA reaches high levels, whereas albumin mRNA is barely detectable (2). Reactivation of the AFP gene in the adult is associated with hepatocarcinomas and germ-line tumors (3, 4).

Regulation of AFP synthesis in the mouse has been shown to occur at the transcriptional level (5). Moreover, the 7 kilobase pairs (kbp) upstream of the mRNA initiation site were shown to be necessary and sufficient to direct levels of transcription comparable to those of the endogenous AFP gene in stable integrants of AFP minigenes in F9 teratocarcinomas (6) and transgenic mice (7).

In this paper, we analyze the rat AFP regulatory region by investigating the ability of specific upstream sequences to direct chloramphenicol acetyltransferase (CAT) synthesis in transient expression assays. We have identified three distinct regions involved in hepatic AFP gene regulation. (*i*) A liver-specific enhancer associated with the 5′ end of the AFP gene. (*ii*) The AFP promoter itself works in a tissue-specific manner. (*iii*) Most surprisingly, the overall level of expression is influenced by a tissue-specific, negatively regulating element situated between the enhancer and the promoter. This negative element also has the ability to drastically reduce transcription initiation from heterologous promoters in a tissue-specific manner.

MATERIALS AND METHODS

Plasmid Constructions. λRAF6, containing the 5′ end of the AFP gene, and λRSA30, containing the 5′ end of the albumin gene, were provided by T. Sargent *et al.* (8). pRAF6SR-7 and pRSA30SR-2 are derivatives of pCV1 containing the 3.5-kilobase (kb) *Eco*RI–*Sst* I fragment from λRAF6 and the 5.8-kb *Eco*RI–*Sst* I fragment from λRSA30, respectively. pSV2CAT, pCAT3M, pA10CAT2, πSVx5′Bgl II, and pSVenCAT were provided by L. Laimins (9).

CAT Fusions. The staggered ends of a 2.4-kb *Hpa* II fragment from pRAF6SR-7 (+2 bp to −2.4 kb) were filled using the Klenow fragment of *Escherichia coli* DNA polymerase I, *Bgl* II linkers were added, and after *Bgl* II digestion, the fragment (−2.2 kb to +2 bp) was inserted into the *Bgl* II site of pCAT3M, yielding pAFP2.2CAT. The 5-kb *Eco*RI–*Bgl* II fragment immediately upstream from this 2.2-kb piece was ligated to *Bgl* II linkers and inserted in both orientations into a *Bgl* II partial digest of pAFP2.2CAT to give pAFP7sCAT and pAFP7aCAT. The 5-kb AFP genomic fragment was ligated into the *Bgl* II site of pA10CAT2 in both orientations on the 5′ side of the CAT gene (p5′CATα5s and p5′CATα5a) and in one orientation on the 3′ side of the CAT gene at the *Bam*HI site (p3′CATα5s).

pALB was constructed by inserting a 400-bp *Hin*cII fragment from pRSA30SR-2 after attaching *Bgl* II linkers to the *Bgl* II site of pCAT3M. pALB3′AFP5a was generated by inserting the 5-kb AFP *Bgl* II fragment into the *Bam*HI site on the 3′ side of the CAT gene in pALB. pALB3′SVEN was constructed by inserting the 250-bp *Bgl* II fragment of pSVENCAT containing the simian virus 40 (SV40) 72-bp repeats into the *Bam*HI site of pALB. pAFP5′SVen was constructed by ligating the same *Bgl* II fragment into the −2.2-kb *Bgl* II site of pAFP2.2CAT.

BAL-31 Deletions. pAFP7sCAT was digested with *Sac* I and recircularized to produce pAFP7Δ1. The now unique *Sac* I site was the starting point for internal BAL-31 deletion (10). The BAL-31-digested plasmids were recircularized after addition of *Sal* I linkers to mark the deletion endpoints. The *Bgl* II–*Sal* I enhancer-containing fragment from pAFP7Δ2 was ligated in both orientations into pA10CAT2 to produce p5′CATα3s and p5′CATα3a.

π550. The 550-bp *Hin*dIII–*Sst* I fragment (−4 kb to −3.5 kb) was isolated from pAFP7sCAT, treated with the Klenow fragment, ligated to *Bgl* II linkers, and inserted into the *Bgl* II site of πSVx5′Bgl II (11) in the orientation opposite that found in the AFP upstream region.

Plasmid Preparation. All CAT gene-containing plasmids were transformed into HB101 by the $CaCl_2$ method (12). πVx-based vectors were transformed into MC1061(p3).

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Abbreviations: AFP, α-fetoprotein; kb, kilobase(s); bp, base pair(s); CAT, chloramphenicol acetyltransferase; SV40, simian virus 40.

Cell Growth and Transfection. Approximately 12–16 hr prior to transfection, 10^6 Hepa-1 (13) or CV-1 cells, or 5×10^6 Hep G2 (14) cells were plated onto 10-cm dishes in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal bovine serum. Cells were refed 2–4 hr prior to transfection. Calcium phosphate precipitates were prepared with 30 μg of plasmid DNA per 10-cm plate (9). Twenty-four hours after transfection, fresh media was added to the cells, and cells were harvested 24 hr later.

CAT Assays. Conversion of chloramphenicol to its acetylated forms was as described (15, 16). The activity of each plasmid was determined in triplicate transfection assays. One-tenth the extract from a 10-cm plate was used per assay with incubation times of 10–60 min. This amount of extract gave conversion rates of 1% for pCAT and 2% for pA10CAT2 in 30 min.

RESULTS

The 7 kbp Upstream of the Start Site of the AFP Gene Are Required for Tissue-Specific Expression. To locate the 5′ end of the AFP mRNA, a *Sca* I–*Hpa* I fragment containing sequences of the first (Z) exon (L.M., unpublished data) was 5′-end-labeled at the *Sca* I site and hybridized to yolk sac cytoplasmic RNA. A series of protected fragments ranging in size from 99 to 103 bases was generated after S1-nuclease treatment (Fig. 1). This microheterogeneity, which might result from multiple sites of transcription initiation or from imprecise nuclease digestion, is also observed upon analysis of the 5′ end of the mouse AFP mRNA (19).

To determine sequences required for tissue-specific expression, we inserted the region from −2.2 kbp to +2 bp into the *Bgl* II site of pCAT3M, upstream from the CAT gene. The resulting plasmid, pAFP2.2CAT, was transfected into three different cell lines: Hep G2, Hepa-1 and CV-1. The Hep G2 cell line is derived from a human hepatocarcinoma and synthesizes high levels of AFP and albumin (13). The Hepa-1 cell line, derived from a mouse hepatoma, produces high levels of albumin but little AFP (14). The nonexpressing monkey kidney line CV-1 was used as a control. Surprisingly, no increase in CAT activity above background was detected in any of the cell lines upon transfection with pAFP2.2CAT. However, pAFP7sCAT, which contains the region between −7 kbp to +2 bp of the AFP gene, yielded high levels of CAT activity in Hep G2 cells (Fig. 2*A*) but not Hepa-1 or CV-1 cells.

A Tissue-Specific Enhancer(s) Is Located Upstream from the AFP Site of Transcription Initiation. The necessity of far upstream sequences for efficient AFP transcription suggested the presence of an enhancer element. pAFP7aCAT, where the −7 kbp to −2.2 kbp region was inverted from its natural orientation, yielded even higher levels of activity in Hep G2 cells when compared to pAFP75CAT (Fig. 2*A*).

We tested the ability of this region to activate a heterologous promoter. Its insertion in pA10CAT2, upstream from the SV40 promoter (120 bp upstream from the cap site), increased CAT activity only in Hep G2 cells (see 5′CATα5s, Fig. 2*B*). No increase was observed in CV-1 or Hepa-1 cells (Fig. 2 *A* and *B*). The activation observed in Hep G2 cells was independent of orientation (see 5′CATα5s and 5′CATα5a, Fig. 2*B*). Similar results were obtained when the fragment was placed on the 3′ side of the CAT gene, 1700 bp downstream from the cap site used by the SV40 promoter, or the rat albumin control region extending from −390 bp to +10 bp (see 3′CATα5s, Fig. 2*B* and ALB3′AFP5a, Fig. 2*C*, respectively).

These results demonstrate that the −7 kbp to −2.2 kbp region upstream from the AFP gene contains enhancer elements since it activates transcription independently of position and orientation from heterologous promoters. The element(s) also acts in a tissue-specific manner since it activates the strong SV40 promoter only in Hep G2 cells. Some activation of the weak albumin promoter is observed in Hepa-1 cells (Fig. 2*C*).

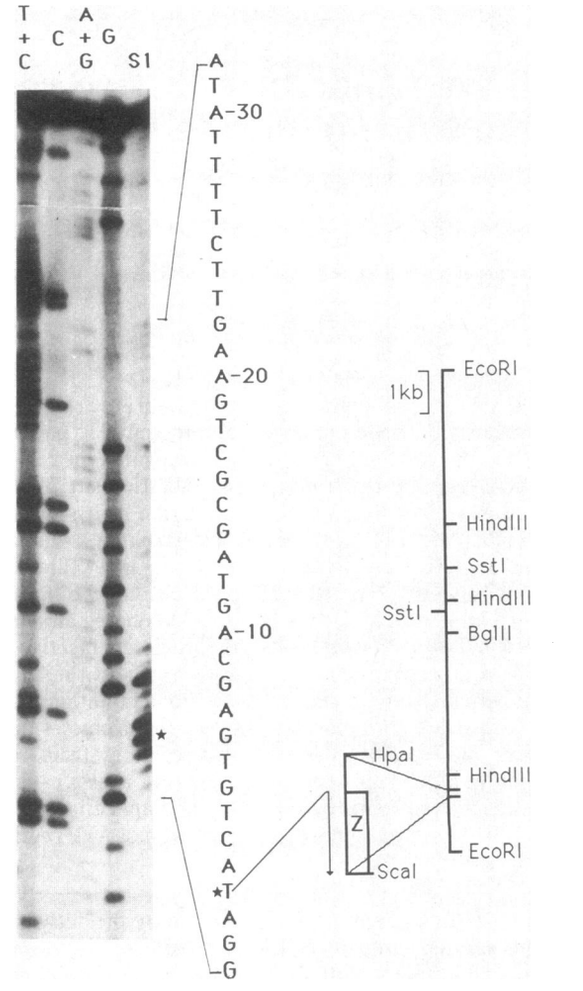

FIG. 1. S1 mapping of the site of transcription initiation of the rat AFP mRNA. (*Left*) The 155-bp *Hpa* I–*Sca* I fragment was 5′-end-labeled at the *Sca* I end, hybridized for 16 hr to 30 μg of yolk sac cytoplasmic RNA from rat fetuses at 16 days of gestation and digested with S1 nuclease (17). The product was analyzed on a 6% polyacrylamide/8 M urea gel together with aliquots of the fragment that had been subjected to Maxam and Gilbert base-specific sequencing reactions (18). The DNA sequence surrounding the sites of transcription initiation (marked with stars) is shown. A canonical "TATA" box is present between −26 and −32. (*Right*) Restriction endonuclease map of the 8.2-kb *Eco*RI fragment encompassing the 5′ end of the rat AFP gene and upstream sequences. The location of the probe used for S1 mapping is shown.

To further localize the enhancer element(s), we constructed internal deletions in the −7 kbp to +2 bp AFP control region. Little change was detected upon deletion of the internal 850-bp *Sac* I fragment (Fig. 3). Further deletions were generated by nuclease BAL-31 digestion from the unique *Sac* I site of the AFP7Δ1. A 5-fold increase in CAT activity was detected upon AFP7FΔ2 transfection. A similar effect was observed when the −7-kbp to −4-kbp portion of AFP7Δ2 was inserted in either orientation on the 5′ side of the SV40 promoter (see Fig. 2*B*, compare 5′CATα3s with 5′CATα5s). Further deletions into the enhancer region decreased CAT activity. AFP7Δ4, containing the distal 1.2 kbp (−7 kbp to −5.8 kbp) of the region, still promotes significant levels of CAT activity. The stepwise decrease in activity observed through the −4 kb- (delimited by AFP7Δ2) to −7-kb region suggests the presence of multiple activating elements.

The AFP Control Region Contains a Tissue-Specific Pro-

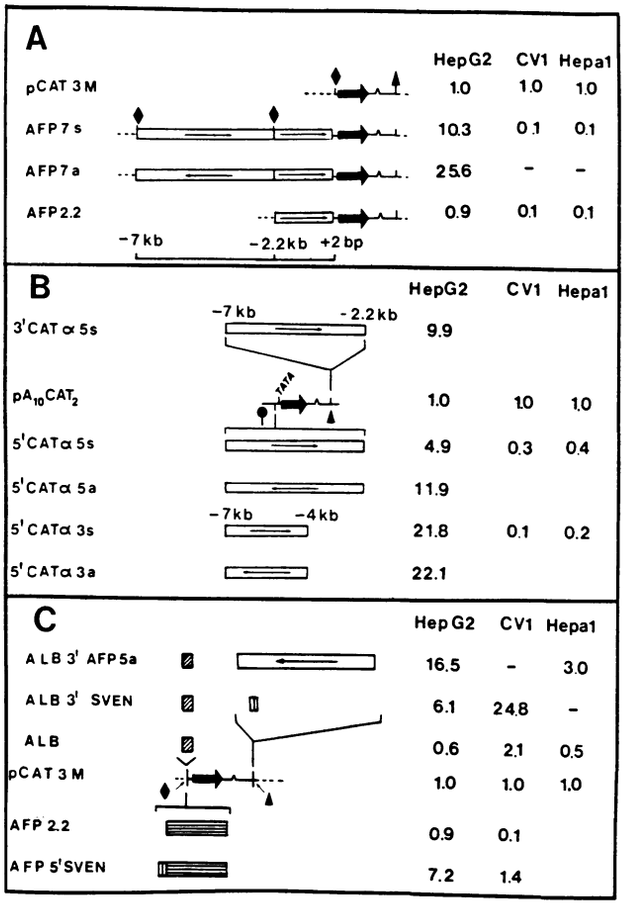

FIG. 2. (*A*) Enhancer–promoter activity of the rat AFP upstream region. Thirty micrograms of each of the vectors shown above were transfected into Hep G2, CV-1, or Hepa-1 cells. (*B*) The rat AFP upstream (−7 kb to −4 kb) region contains a tissue-specific enhancer. The AFP sequences depicted in the figure were inserted into the promoter-containing, enhancerless pA10CAT2 vector. (*C*) The AFP, but not the albumin, promoter is tissue specific. Rat albumin (▨, −390 bp to +10 bp of rat albumin control region) and AFP (═, −2.2 kbp to +2 bp of rat AFP control region) promoter sequences in combination with the SV40 (▯, 72-bp repeats) or AFP (═, −7 kbp to −2.2 kbp of rat AFP control region) enhancers, as depicted above, were tested for activity in Hep G2, CV-1, and Hepa-1 cells. ⟶, orientation of fragment as found in AFP control region. ▲, *Bam*HI; ◆, *Bgl* II; ♦, *Bgl* II–*Spn*. Numbers at bottom represent the coordinates with respect to the rat AFP control region. CAT (➡) activity was normalized to the conversion rate obtained in each cell line transfected with pCAT3M or pA10CAT2.

moter Element. Minimal sequences required for AFP promoter activity were investigated in constructs AFP7Δ6 and AFP7Δ5 that leave 270 bp and 180 bp upstream from the transcription start site, respectively (Fig. 3). Both constructs directed efficient CAT synthesis, demonstrating that the region between −180 bp and +2 bp is sufficient to be recognized as a promoter in Hep G2 cells.

The AFP enhancer was able to activate the albumin (Fig. 2*C*, compare ALB to ALB3'AFP5a) but not the AFP (Fig. 2*A*) promoter in Hepa-1 cells. These results suggested the presence of an additional specificity component in the AFP control region. This was investigated by linking the SV40 enhancer to the AFP −2.2-kb to +2-bp region or to the albumin −390-bp to +10-bp region. Both constructs, ALB3'SVEN and AFP5'SVEN, supported CAT synthesis in Hep G2 cells (Fig. 2*C*). The albumin promoter region driven by the SV40 enhancer was also very effective in directing CAT synthesis in CV-1 cells (Fig. 2*C*). In marked contrast,

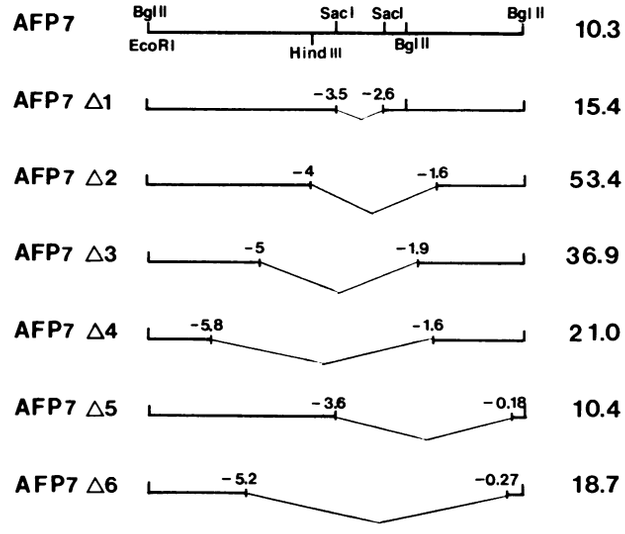

FIG. 3. Deletion analysis of the rat AFP control region. Deletions in pAFP7sCAT were generated. End points of deletions are indicated with respect to the AFP cap site in kbp. CAT activity values were measured in Hep G2 cells and are normalized to the conversion rate of pCAT3M.

the AFP promoter was not activated by the SV40 enhancer in CV-1 cells (AFP5'SVEN, Fig. 2*C*).

The AFP Control Region Contains a Tissue-Specific Negative Regulatory Element. Generation of AFP7Δ2 led to a 5-fold increase in CAT activity with respect to AFP7. This increase in activity could be due to a position effect, to the utilization of a new promoter, or to the removal of a negative regulatory element. Position effects seemed unlikely when we compared the levels of CAT activity induced by AFP7Δ5 and AFP7Δ2 (Fig. 3). AFP7Δ5 places the enhancer(s) closer to the promoter but does not lead to an increase in CAT activity above AFP7.

To show that transcription started at the AFP cap site in AFP7Δ2, we determined the site of initiation by S1 analysis (Fig. 4). The expected 267-base fragment was protected with RNA from both AFP7 and AFP7Δ2 transfections when a uniformly labeled 340-bp *Hpa* I–*Eco*RI fragment, spanning the site of initiation, was used. However, the amount of protected fragment increased drastically with AFP7Δ2 RNA. A low level of readthrough from upstream sequences must occur since a 300-base protected fragment, arising from utilization of a cryptic splice site at position −33, was also detected (20).

We next tested the effect of the putative negative element on the heterologous β-globin promoter activated by the SV40 enhancer. We placed the 550-bp *Hin*dIII–*Sca* I fragment, which comprises sequences that had been deleted in AFP7Δ2 (see Fig. 3), 128 bp upstream from the β-globin cap site in πSVx5'Bgl II in the orientation opposite to that found in the AFP control region. This vector contains the SV40 enhancer downstream from the human β-globin gene. The transcriptional activity of the construct (π550) was detected by S1-nuclease analysis. With the probe used, separate products corresponding to the first and second β-globin exons are resolved (A and B in Fig. 5). Moreover, two other products (A' and A") are detected that arise from the use of cryptic splice sites (21). These sites are only used efficiently in Hep G2 cells. A dramatic decrease in transcriptional activity from the vector containing the negative regulatory element was observed only in Hep G2 cells. In contrast, a slight increase in transcriptional activity was detected in CV-1 and Hepa-1 cells. These results demonstrate that the 550-bp fragment contains sequences that down-regulate transcription in a tissue-specific manner.

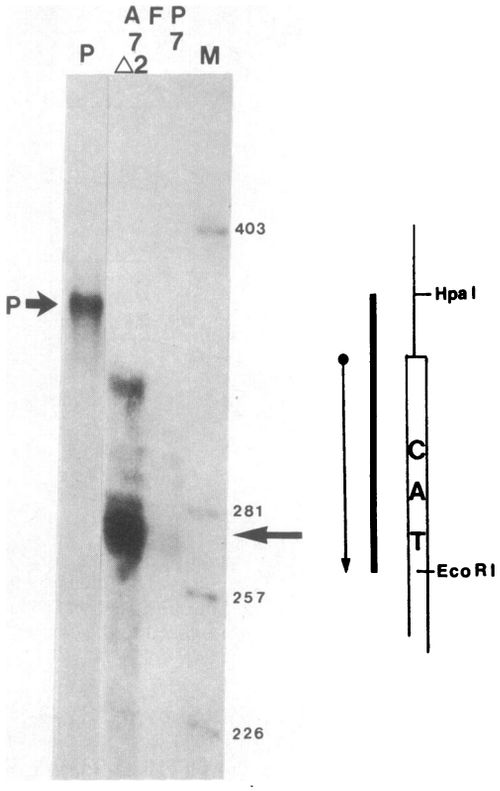

FIG. 4. S1 mapping of the site of transcription initiation in pAFP7sCAT and pAFP7Δ2CAT. (*Right*) The *Hpa* I–*Eco*RI fragment from pAFP7sCAT was cloned in M13mp11 and used to generate a uniformly labeled, single-stranded probe extending from the *Hpa* I site at position −55 to the *Eco*RI site in the CAT gene at position +267. The probe was hybridized to 10 μg of RNA from pAFP7sCAT or pAFP7Δ2CAT transfected cells. (*Left*) The products of S1 digestion were electrophoresed on a 5% polyacrylamide/8 M urea gel. P, probe; M, pBR322 digested with *Alu* I. Arrow marks the migration of the S1 product.

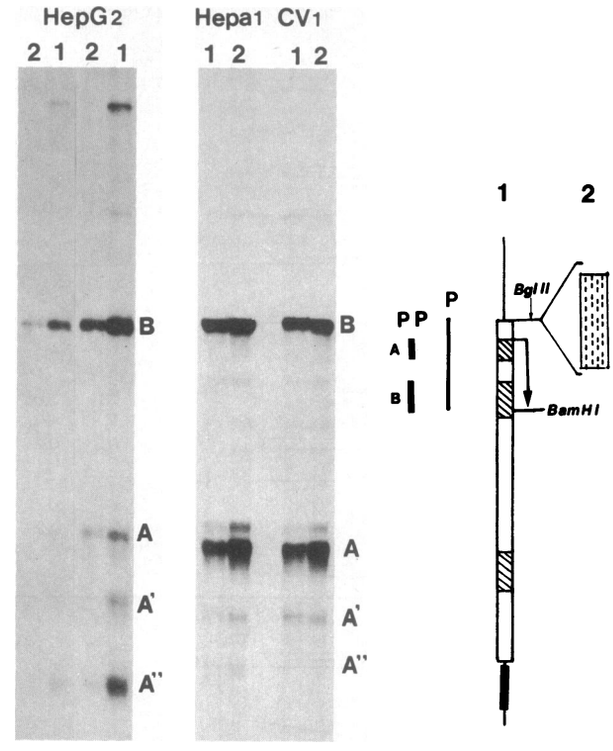

FIG. 5. The rat AFP −3.5-kbp to −4-kbp upstream sequences down-regulate transcription of the β-globin promoter activated by the SV40 enhancer in a tissue-specific manner. The start site and amount of transcription initiation was determined by S1 analysis after transfection of Hep G2, Hepa-1, and CV-1 cells with πSVx5′Bgl II and π550. (*Right*) Relevant features of πSVx5′Bgl II are presented. ▨, β-globin exons. Arrow, start site and direction of transcription. ■, SV40 enhancer. The 550-bp *Hin*dIII–*Sac* I fragment, ▩, present at −4 kb to −3.5 kb in the rat AFP control region was inserted into the *Bgl* II site of πSVx5′Bgl II to yield π550. The *Bgl* II–*Bam*HI fragment (P) was used as a probe. PP, products expected. A, first exon signal. B, second exon signal. (*Left*) S1 mapping. The products of the S1 treatment were separated on 5% polyacrylamide/8 M urea gels. A and B are as described above. A′ and A″ are products of splicing to cryptic donor splice sites in the first exon (21). Two exposures of the autoradiogram are shown for Hep G2. 1, πSVx5′Bgl II transfection. 2, π550 transfection.

DISCUSSION

We have used transient expression assays to define the control region of the rat AFP gene. The reporter gene CAT was fused to upstream sequences flanking the rat AFP gene. Recombinants were introduced into AFP- and non-AFP-producing hepatic cells (Hep G2 and Hepa-1) and nonhepatic cells (CV-1). Surprisingly, the upstream 2200 bp were unable to direct CAT expression in an AFP-synthesizing, human hepatoma cell line (Hep G2). High levels were detected only after inclusion of the 7-kb upstream region of the rat AFP gene (Fig. 2*A*).

The differential behavior of the 2.2-kb vs. 7-kb AFP upstream region demonstrated the existence of a far upstream activating element. The large distance between the activating sequences and the site of transcription initiation, its orientation-independent activity (Fig. 2*A*) relative to the direction of transcription when placed upstream or downstream of the gene (Fig. 2*B*), and its ability to activate heterologous promoters (Fig. 2) strongly indicates the presence of an enhancer (22, 23). However, these sequences cannot activate transcription in CV-1 cells indicating that they are recognized by tissue-specific factors, a property that is shared by most cellular enhancers thus far described (24–27). This is in contrast with the findings of Godbout *et al.* (28) that showed activation of the herpes thymidine kinase promoter by the mouse AFP enhancer not only in Hep G2, but also in Hela cells. We see no activation of the SV40 promoter by the rat AFP enhancer in Hela cells (data not shown).

The location of the enhancer containing region between the albumin and AFP genes (29, 30) suggests a reason for the conservation of the linkage of the two genes during evolution. In contrast to a report (31), we find inefficient utilization of the 400-bp albumin upstream region (−390 bp to +10 bp) as a promoter (Fig. 2*C*). However, inclusion of the AFP enhancer 3′ to the albumin promoter significantly increases activity (Fig. 2*C*). At certain stages during development or in a given tissue, the AFP enhancer could serve a similar function *in vivo*. Although the enhancer would be 20–25 kb away from the albumin promoter, there is a precedent for enhancer function from this distance (32).

Deletion analysis of the −2.2- to −7-kbp region revealed that activating sequences are present 6–7 kb upstream from the site of transcription initiation (AFPΔ4). Moreover, the gradual decay in activation potential observed in AFP7Δ2, Δ3, and Δ4 constructs indicates that activating sequences are not restricted to the utmost upstream 1200 bp of control region DNA. Indeed, they span 3 kbp located between −4 kbp and −7 kbp upstream from the transcription initiation site. The enhancing elements that have been described for the mouse AFP gene lie between −1.0 kbp and −7.6 kbp (28). The extent of the AFP enhancer sequences indicates the presence of a complex element. It would not be surprising to find multiple repeating sequence elements characteristic of

complex enhancers such as those associated with the cytomegalovirus (33) and β-interferon (34) enhancers.

The inability of AFP promoter to undergo SV40 enhancer-mediated transcription activation in CV-1 cells argues for additional tissue specificity associated with regions proximal to the transcription initiation site. This has been described for the insulin (35) and immunoglobulin (36, 37) promoters. In contrast, the albumin "promoter" (−390 bp to +10 bp) is capable of high level activation by the SV40 enhancer in CV-1 cells (Fig. 2*C*). At present, we do not know the significance of the differential behavior of the albumin and AFP promoters.

Certainly, the most unexpected finding of these studies is the presence in the rat AFP control region of tissue-specific, cis-acting negative regulatory sequences. This element, located approximately 3.5 kb upstream from the transcription initiation site between the enhancer region and the promoter, shares the following several properties of enhancers: it can act over a large distance, as observed in the AFP control region, and in an orientation-independent manner as observed in π550. Repression of transcription from the β-globin promoter (Fig. 5) demonstrates that it can act on heterologous promoters. This down-regulation occurs at the initiation site normally used by the linked promoter (Figs. 4 and 5), not merely by suppression of readthrough transcription from cryptic vector promoters.

At the present time, there are relatively few examples of eukaryotic negative regulatory elements. The yeast (38) and rat repetitive DNA (11) "silencer" elements have characteristics very similar to the AFP negative regulatory sequence in their ability to down-regulate transcription. Unlike the rat repetitive DNA silencer, the AFP negative regulator acts in a tissue-specific manner, suggesting the action of trans-acting factors in repression.

The combination of three cell-type specific elements, a promoter, a negative regulator, and a complex enhancer (as they exist in the rat AFP control region) provides a solution to the problem of synthesis of a product not only in different tissues but also at different rates during developmental stages. We would like to speculate that tissue-specific activation of AFP transcription is mediated through the binding of trans-acting factors to the enhancers and promoter elements to yield stable transcription complexes (39, 40). We postulate that the binding of a repressor to the negative regulatory element is responsible for the perinatal shut off of AFP synthesis by decreasing transcription from the stably activated promoters. Events leading to positive factor dissociation such as DNA replication and subsequent degradation would preclude the continued requirement for the negative regulator after the initial commitment to repression. The role of the negative element during development is being tested in transgenic mice.

This paper is dedicated to Dr. Luis F. Leloir on the occasion of his 80th birthday, September 6, 1986. We thank Drs. G. Khoury and L. Laimins for their hospitality towards one of us (L.M.), Drs. L. Laimins and A. B. Chepelinsky for their patience, advice, and productive discussions, and Dr. E. Goldwasser for allowing us to use his tissue culture facilities. L.M. was a 1984–1985 Roesing Scholar and a Medical Scientist National Research Service awardee. This work was supported by funds from the Cancer Research Foundation, by an ACS International grant to L.M., and by Grant R01 AI 12575 from the U.S. Public Health Service to L.B.R.-D.

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