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Multiple Regulatory Elements in the Intergenic Region between the α-Fetoprotein and Albumin Genes

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Three enhancer elements spanning a distance of 7 kilobases have been found at the 5′ end of the α-fetoprotein (AFP) gene. These elements were identified by transient expression assay after the introduction of a modified mouse AFP gene with variable amounts of 5′ flanking sequence into a human hepatoma cell line, Hep G2. These regulatory elements function in a position-independent and orientation-independent manner that is typical of enhancers. All three elements will stimulate transcription from the promoter of the herpes simplex virus thymidine kinase gene. In Hep G2 cells, transcriptional activation from the heterologous promoter was approximately 25- to 50-fold higher than the basal levels obtained in the absence of AFP enhancer elements. In HeLa cells, the increase in thymidine kinase gene transcription varied from 6- to 14-fold, indicating that the enhancer elements exhibit some cell type specificity. Deletion analysis of the region proximal to the AFP transcription initiation site identified an essential region between 85 and 52 bases upstream of the site of initiation of transcription whose removal resulted in almost complete extinction of transcriptional activity. This region, which has been shown to be dispensable for transcription in HeLa cells, defines a second tissue-specific regulatory region in the gene.

The α-fetoprotein (AFP) and albumin genes form a small gene family which diverged 300 to 500 million years ago (25, 32, 33, 35, 40), yet in both the human and mouse genomes, they have remained tightly linked, separated by an intergenic region of approximately 14 kilobase pairs (kb) of DNA (31, 63). The AFP and albumin genes are coactivated in mouse embryonal development in the visceral endoderm of the yolk sac and several days later in the fetal liver and gastrointestinal tract (16, 17, 61, 67). Shortly after birth, expression of the AFP gene in both the liver and gut is turned off, with levels of mRNA in the liver reduced by 10^4-fold 3 to 4 weeks postnatally (4, 61). In contrast, albumin gene expression remains high in the liver and declines only in the gut. If, as has been suggested (61), these genes have remained tightly linked to share regulatory elements, these elements would be important in determining tissue specificity but almost certainly could not be involved in the maintenance of absolute transcription rates, as these two genes vary markedly in levels of transcription.

By stable DNA-mediated transformation of F9 teratocarcinoma cells with a modified AFP gene containing the entire 14-kb intergenic region, we showed that this region contained sufficient DNA information to confer tissue-specific expression of the introduced gene upon differentiation to visceral endoderm (56). In both F9 cell transformants (T. Vogt and R. Scott, unpublished results) and in transgenic mice carrying similar modified AFP genes (37), 7.6 kb of the intergenic DNA immediately 5′ of the AFP gene was sufficient to direct tissue-specific expression and developmental regulation from the introduced DNA. In this report, we describe a finer mapping of regulatory elements by the transient introduction of genes into tissue culture cells. Transient expression assay systems have proved to be an efficient and accurate way to delineate proximal and distal regulatory sequences responsible for correct initiation and modulation of RNA transcription (10, 19, 28, 65). Since the DNA is not incorporated into the genome, variations associated with random chromosomal integration of DNA are avoided.

By transiently introducing the AFP genes into a human hepatoma cell line, Hep G2, three elements have been identified between −1.0 and −7.6 kb which enhance transcription from the AFP promoter by at least a factor of 20. All three elements fulfill the general criteria for enhancer elements; i.e., they are orientation and position independent. They also stimulate transcription from the heterologous herpes simplex virus (HSV) thymidine kinase (TK) promoter in Hep G2 cells and to a lesser extent in HeLa cells, indicating that the AFP enhancer elements are not strictly tissue specific.

In addition to these distal regulatory elements, one proximal tissue-specific regulatory sequence upstream of the TATAA box was identified between −85 and −52 base pairs (bp) of DNA upstream of the start of transcription.

MATERIALS AND METHODS

Construction of AFP and HSV TK minigene plasmids. The AFP minigene, ZE, was constructed by joining in pBR322 two genomic *Eco*RI fragments from the AFP gene, producing a five-exon AFP gene containing the first three and last two exons of AFP, 7.6 kb of 5′ flanking DNA, and 400 bp of 3′ flanking DNA (56). The 5′ deleted and internally deleted constructs were obtained by cutting ZE at convenient restriction enzyme sites and religating it into pBR322. When necessary, linkers were added by blunt-end ligation to the filled-in ends of fragments generated by restriction enzyme cleavage. The linked fragments were separated from free linkers by RPC-5 chromatography (62), and the DNA was digested with an excess of restriction enzyme and ligated to plasmid DNA. Transformation of *Escherichia coli* HB101 and plasmid DNA isolation was done by standard techniques. Thymidine kinase plasmids were constructed by blunt-end ligation of *Sal*I linkers to filled-in free ends of a *Pvu*II digest of the HSV TK gene (41). This TK fragment was ligated into the *Sal*I site of pUC9. Elements I, II, and III were inserted

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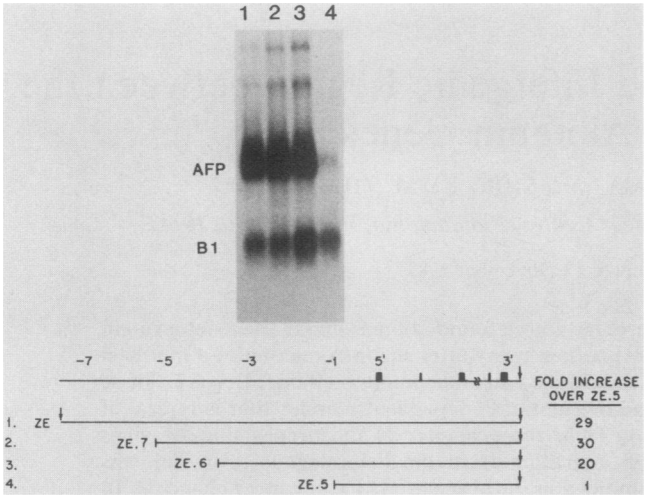

FIG. 1. Deletion analysis of 5′ flanking DNA of the AFP gene by transient expression in Hep G2 cells. Four AFP minigene constructs, containing 7.6 kb (lane 1), 5.3 kb (lane 2), 3.8 kb (lane 3), and 1 kb (lane 4) of 5′ flanking DNA, were introduced into Hep G2 cells by calcium phosphate-mediated DNA transfection. Total poly(A)$^+$ RNA (4 μg per lane), isolated after 48 h, was fractionated by electrophoresis through a 6% formaldehyde–1.5% agarose gel and transferred to a nitrocellulose filter. The bound RNA was hybridized to a denatured nick-translated ^{32}P-labeled DNA probe spanning the first exon and part of the first intron of the AFP gene, including the B1 element (top). The intensities of the AFP and B1 RNA signals were determined by densitometric scanning of autoradiograms. The AFP signal was normalized to the B1 RNA signal and then compared with that of the ZE.5 construct. The values obtained (bottom) are expressed as fold increase over ZE.5 and are the averages of two experiments. The *Eco*RI site (↓) and *Bam*HI sites (|) used to construct the minigenes are indicated (bottom), as are the five AFP coding blocks (■). Distance at the 5′ end of the AFP minigene is indicated by negative numbers representing kilobase pairs.

in both orientations by using the multiple cloning region of pUC9, followed by transformation of *E. coli* JM83. The plasmid DNA used for transfection was banded twice by ethidium bromide-cesium chloride centrifugation. BAL 31 deletions and cloning of resulting fragments were described by Scott and Tilghman (55).

Transient expression assays. Transient expression assays were done as described by Graham and Van Der Eb (27) except that 5×10^6 Hep G2 cells or 1.5×10^6 HeLa cells were plated per 100-mm culture dish in Dulbecco modified Eagle medium–10% fetal calf serum (GIBCO Laboratories). Conditions used for culturing Hep G2 cells also included Ham F12 nutrient mixture in a 1:1 ratio with Dulbecco modified Eagle medium and 10 μg of bovine pancreas insulin (Sigma Chemical Co.) per ml. Fifteen micrograms of DNA per plate was precipitated in calcium phosphate and left on the cells for 12 to 16 h. The cells were harvested 48 h later, and RNA was prepared by the hot phenol method (54) and enriched for polyadenylated [poly(A)$^+$] RNA by oligodeoxythymidylate [oligo(dT)]-cellulose chromatography (2).

RNA analysis of transfected cells. Poly(A)$^+$ RNAs were electrophoresed in a 6% formaldehyde–1.5% agarose gel in MOPS buffer (20 mM morpholinepropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA; pH 7.0) after denaturation of the RNA at 55°C for 15 min in 70% formamide–6% formaldehyde–MOPS buffer. The RNA was then transferred to nitrocellulose filter in 3 M sodium chloride–0.3 M sodium citrate (60), and the filters were hybridized in 50% formamide containing dextran sulfate (64). The DNA probes, ^{32}P-labeled by nick translation, included the following gel-purified fragments: a 660-bp *Hin*cII fragment spanning the first exon of the mouse AFP gene (66), mouse α actin cDNA (43), human AFP cDNA (44), and the 1.9-kb *Pvu*II fragment of the HSV TK gene (41). After autoradiography, the bands were quantitated by densitometric scanning of autoradiograms at different exposures to ensure that the intensity of the signal obtained was directly proportional to the number of ^{32}P-labeled molecules hybridized to each RNA band.

S1 nuclease hybridization assays. The S1 nuclease protection assay to map the initiation site of the AFP transcripts was performed as described by Favaloro et al. (20) and Scott and Tilghman (55). Briefly, the RNA samples were suspended in a hybridization mixture containing 80% formamide, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 5.2], 400 mM NaCl, 1 mM EDTA, and the heat-denatured *Sau*3AI-*Pvu*II probe labeled at the *Sau*3AI site with (γ-^{32}P]ATP (>5,000 Ci/mmol; Amersham Corp.) and polynucleotide kinase. The samples were incubated at 45°C for 16 h and digested with S1 nuclease (*Aspergillus orizae*; Boehringer Mannheim Biochemicals). The samples were precipitated several times with ethanol and suspended in 80% formaldehyde–TBE buffer (90 mM Tris-borate [pH 8.3], 4 mM EDTA)–0.1% bromophenol blue-xylene cyanol, denatured at 90°C for 2 min, and electrophoresed on a 7 M urea–10% acrylamide gel in TBE buffer.

Isolation of plasmid DNA from transfected cells. Forty-eight hours after DNA transfection, cells from one 100-mm culture dish were lysed by the method of Hirt (30). After digestion with proteinase K, the Hirt supernatants were extracted with chloroform and phenol and precipitated in ethanol. Equivalent portions of each sample were digested with *Bam*HI, electrophoresed on a 1% agarose gel in Howley buffer (40 mM Tris-acetate, 1 mM EDTA; pH 7.2) and transferred to nitrocellulose filters (57). Conditions for prehybridization, hybridization, and autoradiography have been described previously (64).

RESULTS

Identification of transcriptional regulatory elements at the 5′ end of the mouse AFP gene. The mouse AFP gene consists of 15 exons spanning a distance of 22 kb. For ease of manipulation, a modified AFP minigene was constructed by joining the first three exons of the AFP gene to the last two exons with 400 bp of 3′ flanking DNA (56). The minigene was then flanked at its 5′ end by 1 kb (pZE.5), 3.8 kb (pZE.6), 5.3 kb (pZE.7), and 7.6 kb (pZE) of 5′ upstream DNA (Fig. 1). These were introduced into the human hepatoma cell line, Hep G2 (36), by calcium phosphate-mediated DNA transfection, and poly(A)$^+$ RNA was isolated 48 h after the addition of DNA. The mouse and human AFP transcripts did not cross-hybridize; in addition, the minigene generated a smaller, polyadenylated, 600-nucleotide-long transcript, allowing its unambiguous identification by Northern blot analysis. One kilobase of 5′ flanking DNA was sufficient to generate a low level of AFP minigene transcription (Fig. 1). However, a 20-fold increase in RNA levels was observed when 3.8 kb of 5′ flanking DNA was present. Additional 5′ upstream DNA did not increase the levels of transcription by more than twofold, indicating that 3.8 kb of 5′ flanking DNA is sufficient to direct high levels of AFP transcription.

The presence of a B1 repetitive element in the first intron of the AFP gene was exploited as an internal control for

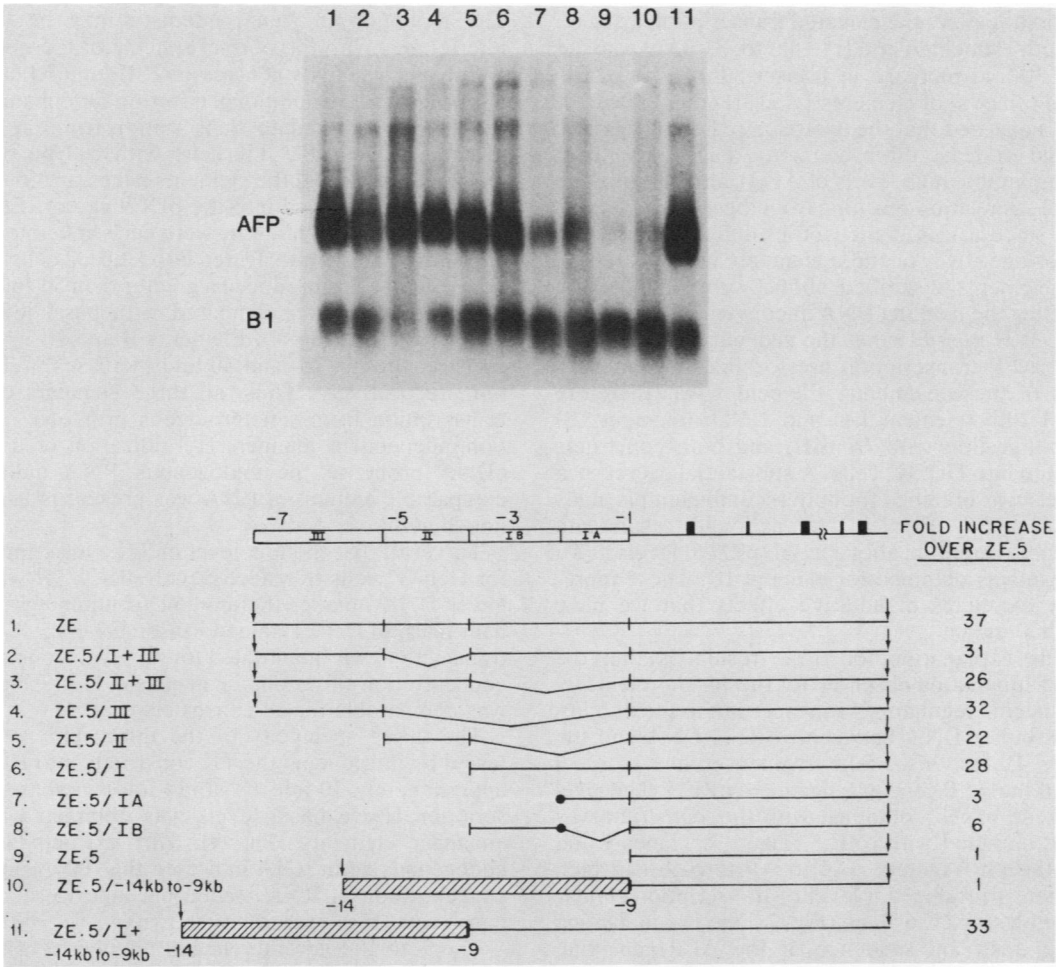

FIG. 2. Identification of multiple regions at the 5′ end of the AFP minigene important for high levels of AFP transcription. Various 5′ flanking DNA sequences were ligated to the ZE.5 construct by using convenient restriction enzyme sites. Element IA includes −1.0 to −2.2 kb, element IB includes −2.2 to −3.8 kb, element II includes −3.8 to −5.3 kb, and element III includes −5.3 to −7.6 kb. Lanes 10 and 11 depict constructs in which the 5′ upstream region spanning −14 to −9 kb was ligated to ZE.5 and ZE.5/I, respectively. Each construct, numbered on the left according to the lanes of the gel, was introduced into Hep G2 cells and analyzed as described in the legend to Fig. 1. The fold increase over ZE.5 obtained for each construct is an average of two to four experiments. Symbols are as described in the legend to Fig. 1 with the following addition: ●, *Hin*dIII restriction sites.

transfection efficiency when various AFP minigene constructs were compared. Transcription of the B1 element, which is transcribed by RNA polymerase III from the opposite strand to that encoding the AFP transcript, has been shown to be unaffected by large variations in the RNA polymerase II directed transcription of the AFP gene (55, 66; T. Vogt and S. Tilghman, unpublished observations). Densitometric scanning of autoradiograms of filters hybridized to a probe which recognizes both the AFP minigene mRNA and the B1 transcript was used to normalize the AFP signal to the B1 signal. Careful internal controls were necessary, as Hep G2 cells, when growing rapidly, express endogenous AFP mRNA at levels of 30 to 40% of that obtained in first-trimester fetal liver. However, when the cells became confluent, a rapid decline in the steady-state levels of AFP mRNA was observed (data not shown). Occasionally, a reduction in the AFP minigene signal relative to the B1 signal occurred, probably as the result of these variations in the growth rate of the hepatoma cell line. Thus, to ensure consistency from experiment to experiment, all constructs to be compared were done at the same time, and pZE and pZE.5 controls were included in all experiments.

Expression of the pZE minigene appears to be entirely cell specific in that no expression was observed in L-cells, HeLa cells, 293 human embryonic kidney cells (26), 1T22 mouse fibroblasts (12), or, in fact, Hepa 1-6 cells (13), a mouse hepatoma cell line which expresses only low levels of endogenous AFP mRNA. Conversely, DNA transfection of primary newborn mouse hepatocytes with pZE, pZE.7, pZE.6, and pZE.5 generated the same pattern of transcription as obtained with Hep G2 cells (R. Godbout, unpublished observations). Thus, the level of expression of the endogenous AFP gene, as well as the tissue origin of the cells, was an indicator of whether a given cell line would efficiently express the gene.

Mapping of the regulatory elements at the 5′ end of the gene by internal deletion analysis. The resection of the 5′ flanking region in Fig. 1 indicated that the region between −1 and −3.8 kb contained a regulatory element necessary for high-level AFP transcription. However, surprisingly, removal of this 2.8-kb region (element I in Fig. 2) from pZE did not significantly lower its level of transcription compared with that obtained with pZE (Fig. 2, cf. lanes 1 and 3). As predicted from Fig. 1, a deletion from −5.3 to −3.8 kb

(element II) did not affect the elevated transcriptional signal. Removal of both elements I and II (−5.3 to −1.0 kb) also did not alter the 30-fold increase in transcription over pZE.5 levels, nor did removal of elements I and III (Fig. 2, lane 5). These results suggested that the upstream region of the AFP gene contained at least three elements, each capable of generating comparable high levels of AFP transcription. The transcriptional stimulation resulting from these elements was not additive, since a less than twofold difference was obtained whether one, two, or three elements were present.

The three elements described above were obtained by arbitrarily cutting the flanking DNA at convenient restriction enzyme sites. It is possible that the sequences responsible for activating AFP transcription are localized within very small regions of these elements. Element I was therefore divided into 1.2-kb (element IA) and 1.6-kb (element IB) fragments by digestion with *Hin*dIII, and both constructs were transfected into Hep G2 cells. A substantial decrease in induction levels was obtained for both recombinant plasmids (Fig. 2, lanes 7 and 8). Element IA was found to stimulate transcription only threefold above basal (pZE.5) levels and a sixfold increase was obtained for element IB. These represent the only examples of additive effects that we have observed in this region.

One possible explanation for these results is that the transcriptional stimulation observed for the various elements is not due to specific regulatory sequences but to their ability to function as buffer DNA between pBR322 DNA and the AFP promoter. To test this, a 5-kb fragment from −14 to −9 kb upstream of the AFP gene was ligated to pZE.5. The level of AFP minigene mRNA obtained with this construct was similar to that obtained with pZE.5 (Fig. 2, cf. lanes 9 and 10). Linking the kb fragment −14 to −9 to pZE.6, which contains element I produced a level of transcription similar to that obtained with pZE.6 alone (Fig. 2, lane 11), indicating that this 5-kb upstream sequence of the AFP gene had neither inhibitory nor stimulatory effects on AFP transcription. The three identified elements therefore appear to activate AFP transcription specifically and do not simply function as buffer DNA.

The distal regulatory elements at the 5′ end of the AFP gene function as enhancers. The three regions of the flanking DNA were then assayed separately for enhancer activity by reversing their orientation at the 5′ end and placing them at the 3′ end of the ZE.5 minigene. No difference in AFP transcriptional levels was observed with element I in either orientation at the 5′ end of the gene (Fig. 3). Separation of element I by approximately 4 kb from the 5′ end of the AFP gene by placing it at the 3′ end resulted in a 13-fold induction over basal level, compared with a 21-fold induction when element I was in its normal position.

Reversing the orientation of element II resulted in a 30% decrease in transcriptional activity (Fig. 3, lanes 6 and 7). Placing it at the 3′ end of the AFP minigene and inserting it in the intron separating exons 3 and 14 in the minigene gave similar results (Fig. 3, lanes 8 and 9). Although element III was not tested for orientation independence at the 5′ end of the gene, its presence at the 3′ end resulted in a 50-fold stimulation in AFP transcription compared with basal levels obtained with pZE.5. This level of transcriptional stimulation was higher than that observed with element III at the 5′ end of the gene (Fig. 3, lanes 10 and 11). It is not known whether this difference is significant. When element III in both orientations was introduced in the third intron of the minigene, significant, although reduced, stimulation of transcriptional activity was obtained (Fig. 3, lanes 12 and 13). The reduction in signal intensity may be the result of interference with the normal splicing of the minigene.

Enhancing activity of elements I, II, and III on a heterologous promoter. An additional criterion for enhancer activity is the ability to stimulate transcription from an heterologous promoter. The HSV TK gene with 200 bp of 5′ flanking DNA, containing all the elements necessary for its transcription (42), was cloned into the pUC9 vector. Elements I, II, and III in both orientations were each linked to the TK gene and subsequently transfected into Hep G2 cells. Element I in both orientations produced a greater than 50-fold stimulation of TK transcription compared with basal levels obtained with pUC9 TK (Fig. 4). Elements II and III in both orientations resulted in 25- and 40-fold increases in TK transcription, respectively. Thus, all three elements can stimulate transcription from a heterologous promoter in an orientation-independent manner. Hybridization of a human AFP cDNA probe to the endogenous RNA indicated that a comparable amount of RNA was present in each lane (data not shown).

To verify that the low level of TK transcription observed for Hep G2 cells transfected with pUC9 TK was not due to lower DNA uptake, the amount of nonintegrated recombinant plasmid DNA retained in Hep G2 cells 48 h after DNA transfection was quantitated for all seven constructs (1). The concentration of recombinant plasmids present in the cells was comparable for all constructs (Fig. 4).

The tissue specificity of the three AFP enhancers was tested by introducing the TK constructs into HeLa cells. An approximately 10-fold (6- to 14-fold) increase in TK transcription above basal levels was obtained with the three enhancer elements (Fig. 4). Hirt extracts and levels of endogenous actin RNA indicated that DNA uptake and the concentration of RNA loaded per lane were similar for all constructs (Fig. 4; data not shown).

The 5- to 10-fold difference in the degree of enhancement of the three elements in Hep G2 cells versus that in HeLa cells cannot be attributed to a greater general efficiency of enhancer-dependent transcription in the former, as similar constructs in which the simian virus 40 enhancer replaced the AFP elements showed the opposite effect, that is, a larger response in HeLa cells than in Hep G2 cells (data not shown). Thus, these results indicate that the elements exhibit some degree of tissue specificity but are recognized by factors present in HeLa cells. This is in contrast to the immunoglobulin (3, 23, 45) and insulin (17a, 65) enhancers, which exhibit far more stringent specificity.

Identification of a proximal tissue-specific regulatory sequence necessary for AFP transcription. Previous transient expression assays in HeLa cells demonstrated that only the TATAA box at −30 bp was required for AFP minigene transcription when driven off the SV40 enhancer (55). The apparent absence of any promoter element other than the TATAA box was in striking contrast to results obtained with many other genes, for which sequences between −120 and −40 bp were required for efficient transcription (5, 15, 18; 42). To determine whether promoter-proximal regulatory sequences were unnecessary in Hep G2 cells as well, we used BAL 31-generated AFP minigene constructs with *Bam*HI-linked endpoints at −600, −118, −85, −52, and −33 bp. The AFP enhancer element I was placed at the 5′ endpoint, and the resulting minigenes were introduced into Hep G2 cells. No significant reductions in transcriptional levels were observed between pZE.5/I, which has no internal deletion, and the two constructs with deletions up to −600 and −118 bp, respectively (Fig. 5). Deletion of se-

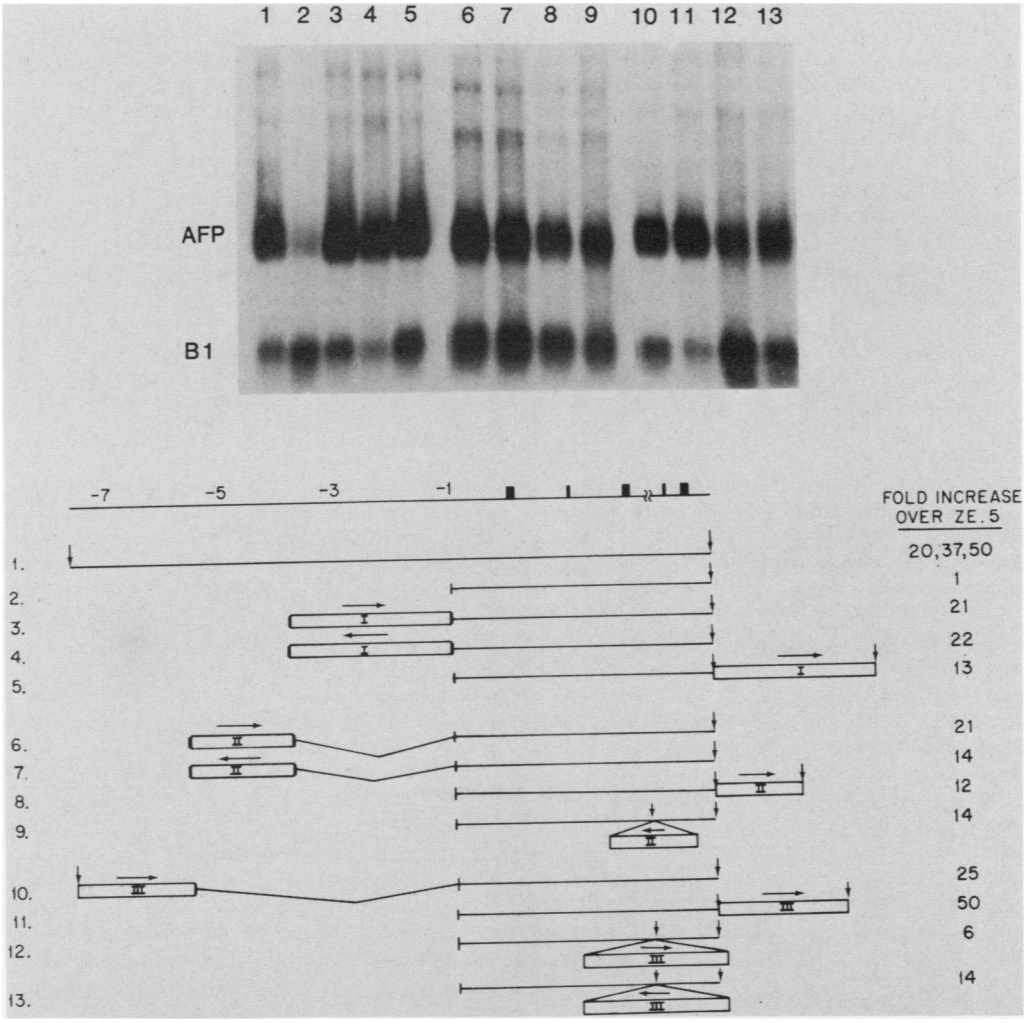

FIG. 3. Regulatory elements at the 5′ end of the AFP gene function as enhancers. Elements I, II, and III (described in Fig. 2) were analyzed for enhancer activity by transient expression assay and Northern blot analysis as described in the legend to Fig. 1. The constructs are numbered on the left according to the lanes of the gel. In some cases, for example, when elements were placed at the 3′ end of the gene and element II and III were placed within the gene itself, *Eco*RI linkers were ligated to one or both ends of the DNA fragment. Symbols are as described in the legend to Fig. 1, with the following addition: horizontal arrows indicate the orientation of the regulatory elements. Since the three elements described were each analyzed separately in an experiment which included both ZE and ZE.5 controls, and since the fold induction can vary from experiment to experiment, values obtained with the ZE constructs in the three experiments are listed separately. The first number listed, 20, is to be compared with values obtained with element I; the second number is to be compared with values obtained with element II constructs, and the third number is to be compared with element III constructs. The values listed for lanes 1 to 10 represent an average of two or more experiments. The last three constructs were tested only once.

quences up to −85 bp, resulted in an approximately twofold reduction in transcriptional activity. Deletion of an extra 33 bp (to −52 bp) produced a dramatic decrease in transcriptional activity, to <1% of pZE.5/I. Comparable results were obtained by reversing the orientation of element I (Fig. 5, lanes 3, 5, 7, 9, and 11).

To verify that the AFP minigene transcripts in Fig. 5 initiated at the correct site, they were hybridized to a DNA probe extending from the untranscribed 5′ end of the minigene to a ^{32}P-labeled *Sau*3AI site at position +56 in the first exon. Correct initiation was obtained in constructs with at least 85 bp of 5′ flanking DNA (Fig. 6). No correctly initiated transcripts could be detected with templates containing only 52 or 33 bp of upstream DNA, although several other minor bands were evident.

Transfection of HeLa cells with pZE.5, the plasmid without enhancers, as well as with plasmids, which contained one or all three of the enhancer elements, generated B1 transcripts but no detectable AFP transcripts (Fig. 5, lanes 12 and 13). Since the AFP enhancer elements are active in HeLa cells in conjunction with the TK promoter, the strict tissue specificity observed for AFP expression must not reside in these elements alone; rather, tissue-specific function must reside in the proximal regulatory region between −85 and −33 bp or within the minigene itself or both. Alternatively, factors in HeLa cells could recognize this promoter element, but further upstream DNA could act as a suppressor element in heterologous cell types. However, removal of DNA between −1000 and either −118 or −85 bp from a AFP minigene construct which has element I (Fig. 5, lanes 14 and 15) did not relieve the block in AFP transcription in HeLa cells, indicating that if there is suppressor DNA, it is not found within this region.

The likelihood that tissue-specific factors may be required

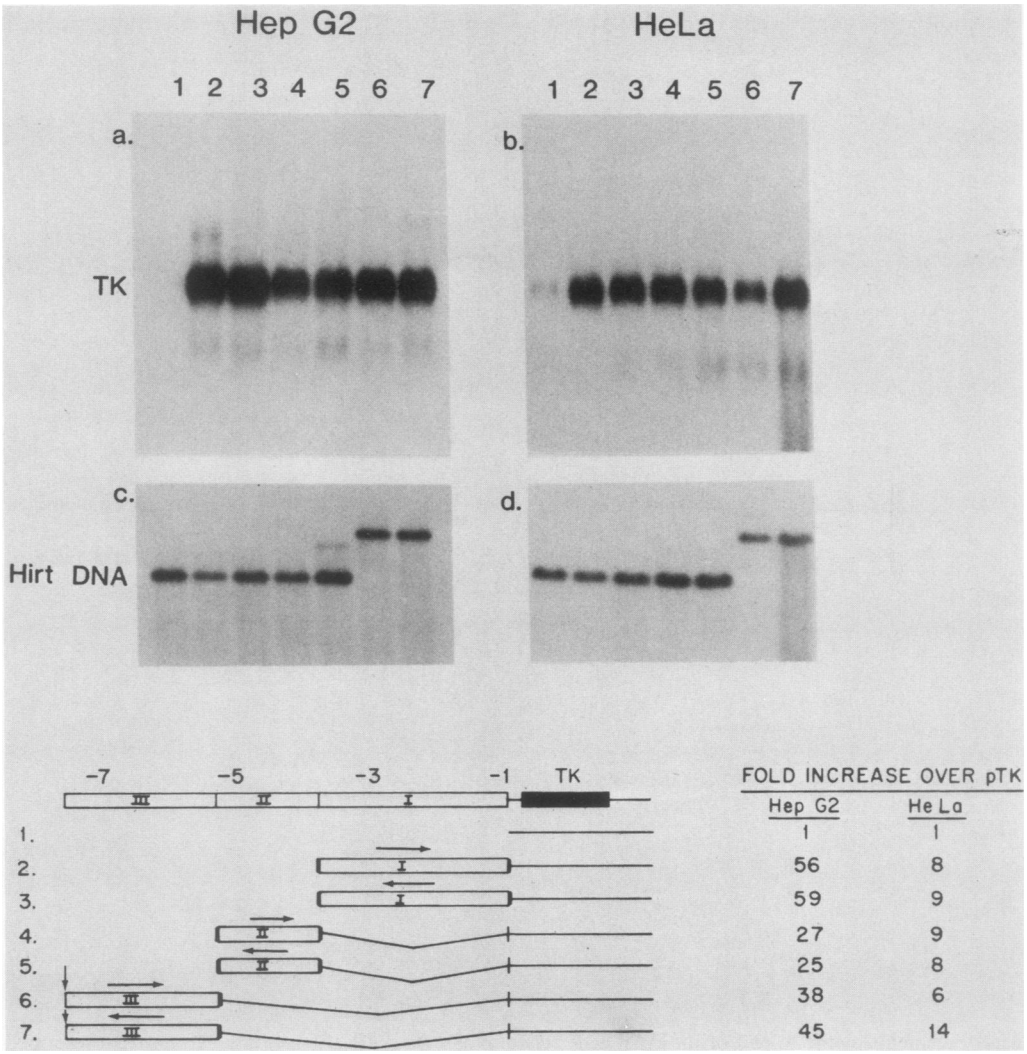

FIG. 4. Effect of elements I, II, and III on a heterologous promoter. The HSV TK gene, containing 200 bp of 5′ flanking DNA, was introduced in the *Sal*I site of the multiple cloning region of pUC9 after ligation of *Sal*I linkers to a *Pvu*II digest of herpes simplex virus TK DNA (41). Elements I and II were inserted into the pUC9 TK plasmid at the *Bam*HI site in both orientations. Element III was introduced into pUC9 TK at the *Bam*HI-*Eco*RI site. In this case, the TK gene was inserted in both orientations instead of element III. DNA transfection of Hep G2 and HeLa cells was as previously described, with the exception that 10 μg of plasmid DNA was used per plate. Poly(A)$^+$ RNA was isolated, and 3 μg was loaded in each lane of a 1.5% agarose–6% formaldehyde denaturing gel. The RNA was transferred to a nitrocellulose filter and hybridized to nick-translated ^{32}P-labeled TK DNA. Shown are Northern blot analyses of Hep G2 cells (a) and HeLa cells (b) transfected with constructs 1 to 7. Plasmid DNA retained 48 h after DNA transfection was isolated by the method of Hirt (30). One-tenth of the low-molecular-weight DNA obtained from one 100-mm plate of cells transfected with each of the seven TK constructs was digested with *Bam*HI and quantitated by Southern blot analysis. Shown are Southern blots of plasmid DNA retained in Hep G2 cells (c) and in HeLa cells (d). Symbols for map (bottom) are described in the legends to Fig. 1 and 3.

for AFP promoter activity is strengthened by an examination of its sequence (Fig. 5). This region has been shown by Scott and Tilghman (55) to contain a series of imperfect inverted repeats that could serve as a binding site for tissue-specific factors and therefore would not be recognized in cells of a heterologous type, such as HeLa cells.

DISCUSSION

In this report, we define two distinct regulatory domains in the 5′ flanking region of the mouse AFP gene, each of which contributes to the tissue-specific expression of the gene in transient expression assays. The first is an enhancer domain between −1.0 and −7.6 kb, which consists of at least three interchangeable elements which behave like classical viral enhancers. That is, they enhance transcription from the homologous AFP promoter as well as heterologous promoters in an orientation- and position-independent manner. The second is a promoter-proximal region between −85 and −33 bp which is tissue specific in the sense that is is entirely dispensable in heterologous cells in the presence of the simian virus 40 enhancer (55) but is absolutely required for transcription in homologous cells.

The AFP gene enhancer domain. Two aspects of the distal enhancer domain are worthy of note: its considerable redundancy and the distance which it spans. The two tandem copies of the simian virus 40 72-bp repeat were the first example of a redundant enhancer, in that simian virus 40 is fully viable with only one copy of the repeat (29, 59). Since

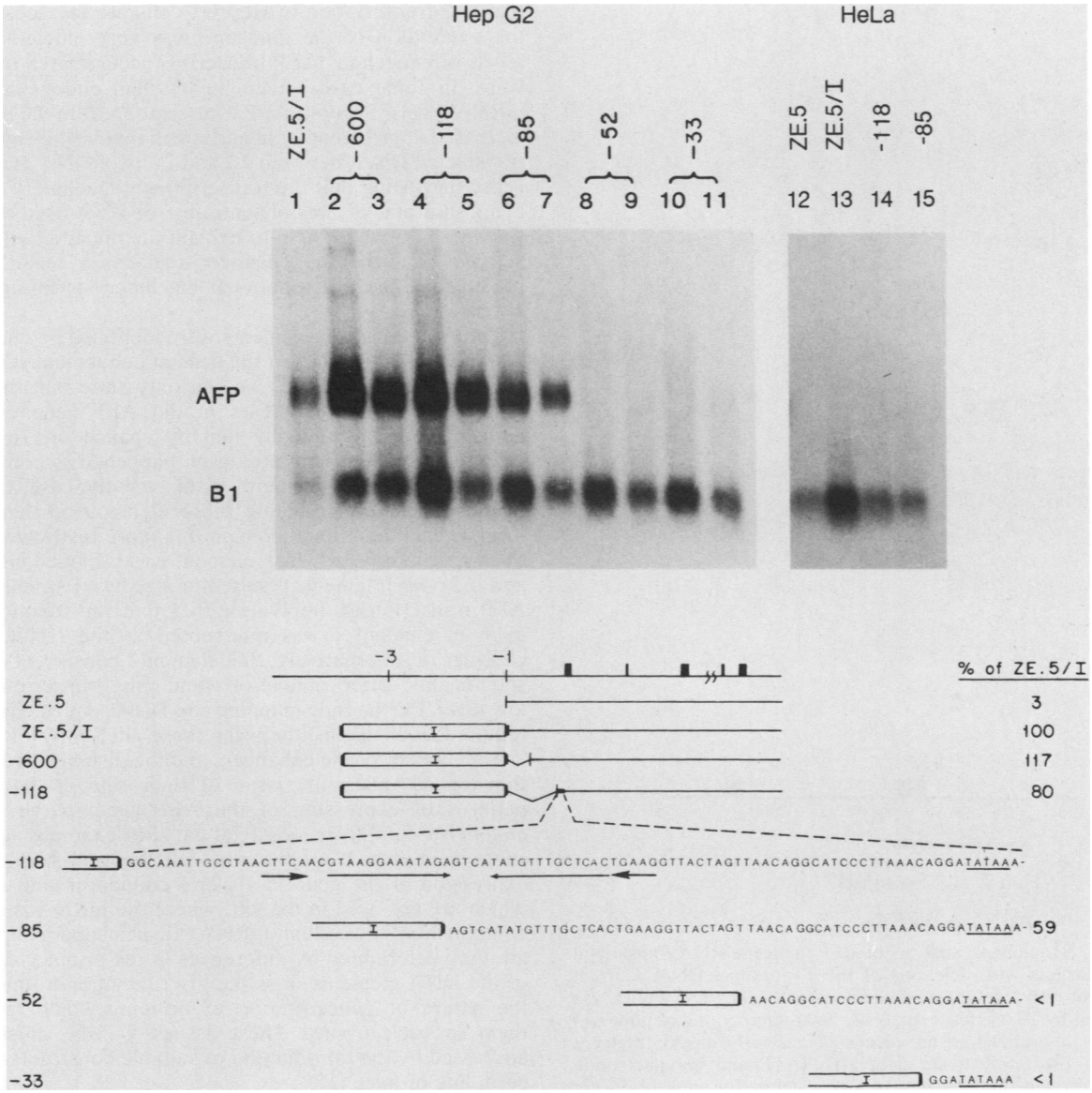

FIG. 5. Analysis of 5′-proximal regulatory sequences. Element I in both orientations was ligated to *Bam*HI-linked ZE.5 minigene constructs with 5′ deletions from −1000 to −600 bp (labeled as −600), −1000 to −118 (labeled as −118), −1000 to −85 (labeled as −85), −1000 to −52 (labeled −52), and −1000 to −33 (−33), respectively. The nucleotide sequence previously described by Scott and Tilghman (55) from bp −118 to −25 is listed to facilitate identification of the endpoints of the deleted DNA; the TATAA motif at −30 is underlined. Imperfect inverted repeats are indicated by arrows. (Top) Each construct was transfected into Hep G2 cells (lanes 1 to 11) or HeLa cells (lanes 12 to 15) and analyzed by Northern blotting and densitometric scanning as described in the legend to Fig. 1. Lane 1 represents AFP and B1 RNA signals obtained for ZE.5/I. ZE.5 RNA was not included in this gel, and the signal intensity was obtained from a different experiment. Results were obtained for the −600 construct with element I in the 5′-to-3′ orientation in lane 2 and in 3′-to-5′ orientation in lane 3. Lanes 4 and 5 represent results obtained for the −118 construct with element I in both orientations. Lanes 6 and 7 represent the −85 construct; lanes 8 and 9 represent the −52 construct. Lanes 10 and 11 represent the −33 construct. The ratio of AFP to B1 RNA signals obtained (bottom) for ZE.5/I is listed as 100%, and values obtained for the other constructs are compared to ZE.5/I. Lanes 12 to 15 represent HeLa cells transfected with pZE.5, pZE.5/I, the −118 construct, and the −85 construct, respectively.

then, multiple, and often interchangeable, regulatory elements have been described in viral genomes, like the immediate early gene of human cytomegalovirus (7) and the glucocorticoid-responsive domain of mouse mammary tumor virus (47), as well as in cellular genes. For example, the heavy-metal domain of the metallothionein gene is repeated several times in the flanking region of that gene (9, 58). Likewise, Goodbourn et al. (24) have recently described a 14-bp sequence in the human β-interferon gene which is repeated five times in the region between −1 and −120 bp. Only three of these repeats are required for maximum virus-induced mRNA transcription in C127 cells. Ryals et al. (53) and Fujita et al. (21) have also identified repeated elements at the 5′ end of the β-interferon and α-interferon genes, respectively. In both cases, maximum gene induction appeared to require all the repeats upon transfection in either L929 or LMTK$^-$ cells. Goodbourn et al. (24) speculate that the number of repeated elements required for maximal

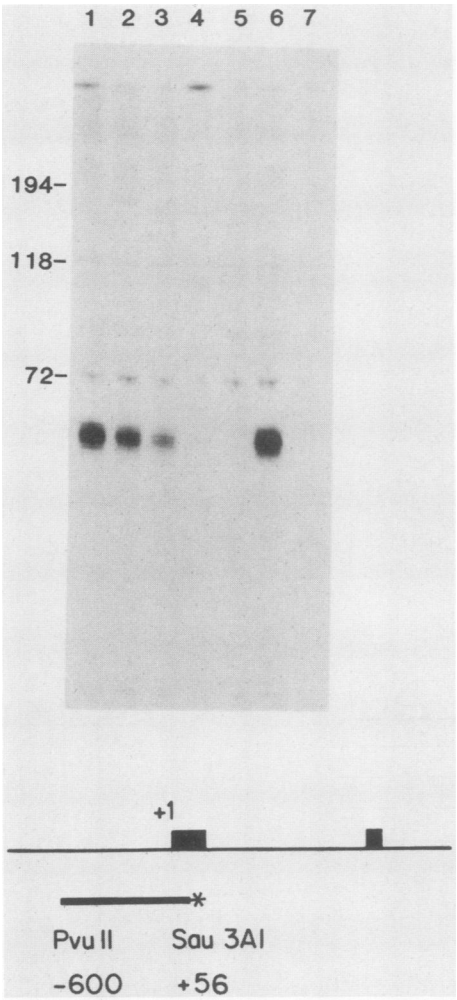

FIG. 6. S1 nuclease analysis of AFP minigene RNA transcribed from constructs with deletions of the 5′-proximal DNA. Correct initiation of transcription from the constructs shown in Fig. 5 was determined by S1 nuclease analysis. Two micrograms of poly(A)$^+$ RNA was hybridized to an excess of a *Pvu*II-*Sau*3AI fragment labeled at the *Sau*3AI site with [γ-^{32}P]ATP and polynucleotide kinase. Correctly initiated transcripts generated a band of 56 bp. Poly(A)$^+$ RNA from −600 (lane 1), −118 (lane 2), −85 (lane 3), −52 (lane 4), −33 (lane 5), and ZE (lane 6) and a tRNA control with no poly(A)$^+$ RNA (lane 7), are represented. Element I was in the 5′-to-3′ orientation. Nucleotide size markers are indicated on the left. A map of the probe, indicating the cap site (+1), labeling site (*), and exons (■), is shown at the bottom of the figure.

expression may depend upon the cell type in which the gene resides.

The significance of the redundancy of regulatory enhancer elements has been elegantly addressed in the case of the *Xenopus laevis* 18S and 28S rRNA genes which are transcribed by RNA polymerase I. In this system, different numbers of short 60- and 81-bp repeats are interspersed in the 5′ flanking region (8, 38, 52). It has been proposed that the 60- and 81-bp elements are attraction sites for factors which are necessary to activate transcription. Genes with different numbers of these repeats will compete equally for transcription when the factors are in excess. However, when the factors become limiting, those copies of the tandem array with more of these elements will compete more effectively.

By analogy, we would argue that the factors responsible for AFP transcription in Hep G2 cells are in excess. First, the expression of the minigene was very efficient, as the levels of exogenous AFP transcripts accumulated over 48 h were, in some cases, even higher than endogenous AFP mRNA levels. Second, AFP transcription from the minigene increased approximately linearly with increasing amounts of transfected DNA, between 0.1 and 15 μg of DNA per 5×10^6 cells, suggesting that the transcriptional machinery was not being saturated at the concentration of DNA used (data not shown). This would help to explain the nonadditivity of the activity of the three enhancer regions, a result that is predicted if the transcriptional machinery is in significant excess.

The AFP enhancer elements were identified by cutting the 7.6-kb 5′ flanking DNA of the gene at convenient restriction enzyme sites. Therefore, we can only state that there is a minimum of three elements in the AFP gene enhancer domain. It seems unlikely that by chance the restriction enzyme fragments generated each happened to contain one individual enhancer element. If this was the case, then one would expect that dividing these elements further would localize each functional domain to a more restricted region. Such an analysis, in which element I was divided into a 1.2- and a 1.6-kb fragment, resulted in a reduced stimulation of AFP transcription, implying either that the functional domain in element I was interrupted as the result of this cleavage or, alternatively, that element I consists of multiple subdomains, each capable of stimulating transcription at a low level. Further fine mapping and DNA sequencing will be required to distinguish between these alternatives.

The number of the enhancers and the distance over which they reside raises the issue of their role in the diverse patterns of expression of the AFP gene in the visceral endoderm, fetal liver, and fetal gut. For example, are there subdomains which are essential for the very high level of expression of the gene in visceral endoderm and liver but which are not used in the gut, where the mRNA is a minor component of the cellular mRNA? If such subdomains exist, are they determined by differences in the primary sequence of the DNA elements or is the discrimination a function of the nature or concentration of proteins which recognize them in each tissue? These issues will be most easily addressed by the introduction of suitable constructs into the germ line of mice (37).

The large size of the AFP enhancer region and its location between the albumin and AFP genes raises the question of whether it affects the expression of the albumin gene in vivo. Should this be the case, it would almost certainly require restricting the activity of the region to the establishment of their common temporal activation during differentiation, as the two genes are regulated in quite different ways in the differentiated cells. Recent transient expression experiments suggest that the region can enhance transcription of an albumin minigene in Hep G2 cells (S. Camper and S. Tilghman, unpublished observations). To do so in vivo, it would have to act over a distance of 25 kb, the distance from the albumin gene promoter to element III.

While there is no demonstrated case of distal elements acting over such a distance, there is no a priori reason why they should not exist. Aside from the large regulatory region described here for the AFP gene, there are several examples now in *Drosophila melanogaster*, where regulatory elements reside as far upstream as 5 kb, in the alcohol dehydrogenase gene (49), and in fushi tarazu (22). In higher eucaryotes, the mouse cytochrome P_1-450 gene has been found to have three functional domains, the most distal of which was between

−1535 and −1265 bp. This region was responsible for induction of gene expression in the presence of an environmental dioxin contaminant (34).

The large size of the AFP gene regulatory region is in marked contrast to that described for two other liver-specific genes. Ciliberto et al. (11) have defined by 5′ resection analysis a 721-bp region of the human α_1-antitrypsin gene which is sufficient to confer liver-specific expression by transient expression assays. It should be noted, however, that only 1,200 bp of 5′ flanking DNA was analyzed, and changing the orientation of the fragment from −1200 to −32 bp resulted in loss of activity. Likewise, Ott et al. (46) described experiments in which a rat albumin gene containing only 400 bp of 5′ flanking DNA behaved in a tissue-specific manner in transient expression assays. In neither case did these authors demonstrate enhancer function. By analogy to the studies described in this manuscript, the regulatory regions identified by these two groups could potentially encompass only the promoter-proximal region of the gene.

Tissue specificity of the AFP regulatory elements. The enhancers of genes with restricted tissue specificity have, in general, been shown to be tissue-specific by DNA transfection experiments. For example, the enhancers found within the mouse immunoglobulin heavy-chain and κ light-chain genes function efficiently only in lymphoid-derived cells (3, 6, 23, 48, 50, 51). In contrast, viral enhancers function in a wide variety of cell types but perform optimally in their natural host cells (14, 39). The AFP enhancers described in this manuscript will enhance transcription, although at a reduced level, from a heterologous promoter in non-AFP-producing cells. These enhancers therefore cannot be considered as entirely tissue specific; general factors found in HeLa cells will recognize these elements as transcriptional activators. It is tempting to speculate that the less strict tissue-specific properties of the enhancers may facilitate diversity in the tissue specificity observed in vivo.

However, transcription from the AFP genes in HeLa cells and a number of other cells of nonhepatic origin is completely undetectable, indicating that the elements responsible for the strict tissue specificity of this gene may reside downstream of the enhancers, i.e., in the promoter or even within the gene itself. The continued absence of AFP transcription in HeLa cells upon removal of upstream sequences beyond position −118 or −85 indicates that lack of transcription is not the result of general suppressor elements between −1000 and −85 bp. The most likely possibility is that tissue specificity is derived from the proximal regulatory sequences upstream of the TATAA box at positions −85 to −52.

The observation that the tissue specificity of a gene is the consequence of multiple and functionally distinct regulatory elements has recently been suggested by several studies. Grosschedl and Baltimore (28) have identified in the mouse immunoglobulin heavy-chain gene sequences in both the promoter and the gene body that contribute to its tissue specificity along with the enhancer. Similarly, the insulin gene promoter as well as the enhancer participate in its pancreatic β-cell specificity (17a). These studies, along with our own on the AFP gene, suggest that multiple regulatory elements, at least some of which are able to function over long distances, contribute in a combinatorial fashion to the strict cell-type specificity observed in vivo. This is especially evident in experiments in which heterologous regulatory elements have been combined to produce an entirely unique tissue specificity, as has been observed in transgenic mice carrying metallothionein promoter-growth hormone gene fusions (59a) or in *D. melanogaster* carrying the SgS4 glue protein gene enhancer and the larval alcohol dehydrogenase gene and promoter (J. W. Posakony and T. Maniatis, unpublished results). In the case of the AFP gene, which is expressed and differentially regulated in at least three tissues, it will be important to determine the relative roles of the distal and proximal elements in each tissue. Finally we should emphasize that we cannot exclude the possibility that additional elements exist in the body of the gene or the 3′ flanking region. However, we have seen no differences in the behavior of minigenes with 5, 8, or 10 of the 15 exons of the authentic gene by either transient expression in Hep G2 cells (data not shown) or stable transformation of murine F9 teratocarcinoma cells (R. Compton and S. Tilghman, unpublished data).

In conclusion, these results demonstrate that there are multiple regulatory elements at the 5′ end of the AFP gene which span a distance of 7.6 kb. The proximal regulatory region appears to be tissue specific, while the distal enhancer elements allow transcription from the HSV TK promoter in heterologous cells, although transcription is reduced by 5- to 10-fold compared with that in homologous cells. This study emphasizes the need to include large segments of flanking DNA when attempting to identify *cis*-acting regulatory elements.

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