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# Liver-specific expression of the mouse α-fetoprotein gene is mediated by cis-acting DNA elements

(transfection/tissue-specific enhancer/tissue-specific promoter/chloramphenicol acetyltransferase)

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ABSTRACT **We have identified cis-acting regulatory elements in the 5′ flanking region of the mouse α-fetoprotein (*Afp*) gene, using the expression of the bacterial gene for chloramphenicol acetyltransferase (CAT) in a transient expression assay. Tissue-specific enhancer activity was determined by transfection of mouse hepatoma (BWTG3) and fibroblast cells (C127, NIH 3T3) with various DNA fragments linked to the CAT gene. A 5.4-kilobase restriction fragment was shown to have characteristics typical of enhancers, including the ability to function independent of orientation and position and the ability to enhance transcription from a heterologous promoter. The enhancer activity was greatest in the hepatoma cells, which express *Afp*. By deletion analysis, it was demonstrated that enhancer activity is present in several subfragments, indicating the presence of more than one element in this fragment. An additional regulatory element within 950 base pairs of the Afp transcription initiation site has been identified and shown to confer tissue-specific expression on the CAT gene. This fragment, which lacks enhancer activity, contains the Afp promoter region and mediates the tissue-specific expression of the CAT gene when driven by nonspecific viral enhancers. We conclude from our studies that there are several types of regulatory elements in the 5′ flanking region of the *Afp* gene that help mediate tissue-specific expression.**

α-Fetoprotein (Afp) is a serum protein that makes up a major portion of the secretory proteins synthesized by mammalian fetal liver cells (1–6). During embryonic development (7–10), liver regeneration (11), and hepatocarcinogenesis (12), this gene is under the control of regulatory processes that involve repression and activation of transcription. In the newborn mouse, for example, transcription is gradually reduced, resulting in a potent developmentally regulated repression of this gene (9, 11). Furthermore, glucocorticoids have been shown to enhance the rate of repression of Afp transcription (13–15). Although the liver is its major site of synthesis, Afp is also synthesized in embryonal yolk sac cells and in the fetal gastrointestinal tract (7, 8, 16, 17). Its synthesis by these cell types is also developmentally regulated (17). In regenerating liver, transcription of the repressed *Afp* gene is activated and upon reconstitution of the liver, transcription is again repressed, as in the developing liver. In hepatocarcinogenesis, however, transcription of the *Afp* gene is activated, and it is stabilized in the transformed hepatocyte (12). The regulation of the *Afp* gene in all of the instances described above occurs at the level of transcription. Recently, it has been demonstrated for several genes that tissue-specific transcriptional control is mediated by cis-acting DNA sequences in the flanking regions of the gene (18–25) or within the transcribed portion of the gene (26–28). In some cases, these sequences appear to be enhancer elements that function best in specific cell types (19, 24, 26–28). Other tissue-specific elements that lack enhancer activity are found in the promoter regions of the rat insulin gene (24) and a mouse immunoglobulin gene (29). The tissue-specific expression of a gene may be mediated by the combined actions of different types of regulatory sequences.

It has been shown that an *Afp* minigene, composed of the 5′ flanking sequences plus the first three and last two exons of the gene, can be expressed in a tissue-specific and developmentally regulated manner in transgenic mice (18) and teratocarcinoma cells (17). Further studies have identified several enhancer elements within a 7-kilobase (kb) region of the 5′ flanking sequences of the *Afp* gene as well as a tissue-specific regulatory region associated with the promoter (30). In this study, we linked *Afp* gene sequences to the bacterial gene for chloramphenicol acetyltransferase (CAT) and analyzed the expression of these recombinants in mouse hepatoma and fibroblast cell lines. We provide further evidence for the cell specificity of the Afp enhancer region and show by enhancer replacement experiment that the Afp promoter region confers cell-specific expression on a bacterial gene.

## MATERIALS AND METHODS

**Construction of Plasmids.** Plasmids were constructed and purified using standard protocols (31). All enzymes were purchased from commercial suppliers (Bethesda Research Laboratories, Amersham, and International Biotechnologies). The Afp promoter sequences were derived from a 1-kb *Bam*HI/*Sau*3AI fragment that contains ≈950 base pairs (bp) of 5′ flanking sequence and 52 bp of the first exon (32). This fragment was treated with BAL-31 to remove the coding sequences present in the first exon and ligated into the *Hin*cII site of pUC8. We determined by DNA sequencing that the deletion end point is at position +37 in the untranslated portion of the first exon. The plasmid BSDcat was constructed by removing the fragment from pUC8 by digestion with *Bam*HI and *Hin*dIII and inserting it into *Bam*HI/*Hin*dIII-cut pP5cat (a gift from Scott C. Supowit). In this vector, derived from pSV2cat (33), the *Bam*HI site 3′ to the CAT gene has been deleted, and another *Bam*HI site was created 5′ to the *Hin*dIII site of pSV2cat.

The upstream Afp sequences are contained in a *Pvu* II fragment that extends from about −600 bp to −7 kb. Our genomic clones are missing 1 kb of DNA from −2.8 kb to −3.8 kb so the *Pvu* II fragment we used is 5.4 kb. This missing piece of DNA is at the *Eco*RI site (Fig. 1), which was generated artificially during the cloning procedures. The *Bam*HI/*Pvu* II fragment of BSDcat was removed and the *Pvu*

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Abbreviations: Afp, α-fetoprotein; CAT, chloramphenicol acetyltransferase; Mo-MuLV, Moloney murine leukemia virus; kb, kilobase(s); bp, base pair(s); SV40, simian virus 40.

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II fragment was inserted after filling in the protruding end generated by *Bam*HI using the large fragment of DNA polymerase I. Both orientations of the *Pvu* II fragment were prepared (Afp-CAT and Inv-Afp-CAT). The *Pvu* II fragment was inserted into the *Bam*HI site of pSV1cat (33) after filling in the protruding ends generated by *Bam*HI.

The simian virus (SV40) enhancer was derived from pSV2cat (33) by digestion with *Acc* I and *Nco* I. This fragment was cloned into BSDcat cut with *Acc* I and *Bam*HI after filling in the ends generated by *Nco* I and *Bam*HI. The Moloney murine leukemia virus enhancer (Mo-MuLV) is contained in a *Bam*HI fragment (a gift from Inder Verma) and was inserted into the *Bam*HI sites of BSDcat and pSV1cat.

Deletion mutants were derived from Afp-CAT. After digestion with the enzymes indicated in Fig. 5, the non-complementary ends were filled in and the plasmids were religated. The deletions were confirmed by mapping with restriction enzymes.

**Cell Culture and Transfections.** The BWTG3 cell line is derived from a mouse hepatoma and continues to express many hepatic proteins as well as Afp (34). The mouse cell lines C127 and NIH 3T3 do not synthesize Afp. All cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% bovine calf serum (HyClone, Logan, UT). One day before the transfections, the cells were seeded at $10^5$ cells per 10-cm dish. Transfection was done by precipitating 20 $\mu$g of DNA with calcium phosphate (35) in a vol of 1 ml and adding it to each dish. The DNA was removed 4 hr later and the cells were treated with 15% (vol/vol) glycerol for 2 min before being refed. In all experiments, 1.5 pmol of plasmid DNA was used and salmon sperm DNA was added to 20 $\mu$g (1.5 pmol of pSV2cat corresponds to 5 $\mu$g). Cells were harvested 48 hr later and CAT assays were performed as described (32). Reaction mixtures were incubated for 1 hr at 37°C and contained 0.2 $\mu$Ci of [$^{14}$C]chloramphenicol (1 Ci = 37 GBq) (New England Nuclear) and one-fifth of the cell extract from one plate. The reaction products were separated by thin-layer chromatography and visualized by autoradiography. For comparison of different samples, the spots were cut out and counted. The concentration of protein in the cell extracts was determined by the method of Bradford (36) and activities were expressed as pmol acetylated per hour per mg of protein.

## RESULTS

**Afp cis-Acting Elements Exhibit Tissue-Specific Promoter Activity.** To identify the cis-acting control elements of the *Afp* gene, we linked a 1-kb fragment of the *Afp* gene to the bacterial CAT gene coding sequences (Fig. 1, BSDcat). This fragment includes 37 bp of the first exon, the Afp promoter region including the TATA box (32), and ≈950 bp of 5′ flanking sequence. When transfected into the BWTG3 (hepatoma), NIH 3T3, and C127 cells, this plasmid (BSDcat) is expressed at a low level in all three cell types—i.e., 1–4% of the activity of the control plasmid pSV2cat (Fig. 1, lanes 2; Table 1). There appears to be a slightly higher (2-fold) activity, relative to pSV2cat, in BWTG3 cells than in the other cell types. The low level of expression of BSDcat indicates that the Afp promoter is able to direct CAT gene transcription, but that no enhancer activity is present. To test for the effects of heterologous enhancers on the Afp promoter, enhancers from SV40 (SV40-BSDcat) and from Mo-MuLV (Mo-MuLV-BSDcat) were inserted into BSDcat (Fig. 1). The SV40 enhancer increases the CAT activity in all three cell types (Fig. 1, lanes 3; Table 1). There are considerable variations in absolute levels of activity between experiments, but within each experiment the activity of SV40-BSDcat relative to pSV2cat is usually higher in BWTG3 cells than in C127 or NIH 3T3 cells (Table 1). When the Mo-MuLV

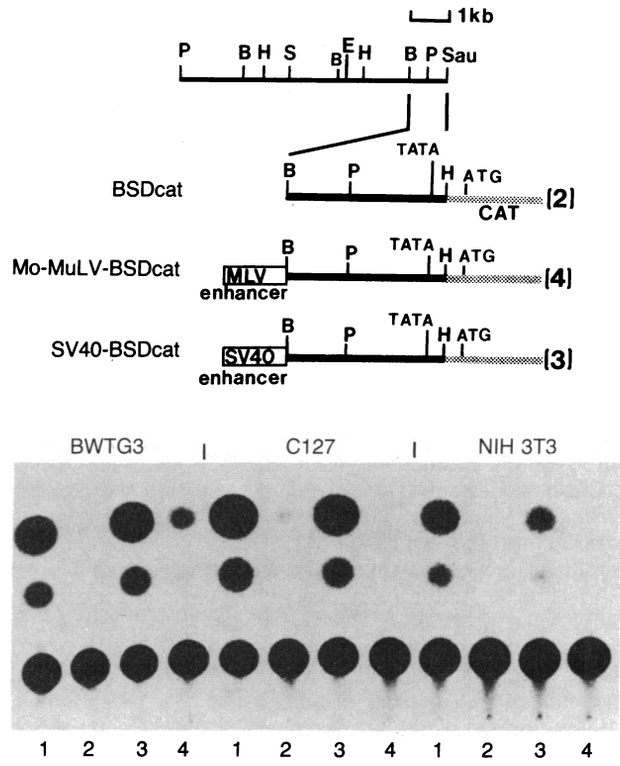


FIG. 1. Construction and expression of the plasmids used to study the proximal regulatory sequences of the *Afp* gene. (*Upper*) The *Bam*HI/*Sau*3AI fragment containing the Afp promoter was converted to a *Bam*HI/*Hin*dIII fragment and joined to the CAT gene as described in *Materials and Methods*. The Afp TATAA box and restriction map are indicated. P, *Pvu* II; B, *Bam*HI; H, *Hin*dIII; S, *Sst* I; E, *Eco*RI; Sau, *Sau*3AI. (*Lower*) Transient expression of the CAT gene in mouse hepatoma (BWTG3) and mouse C127 and NIH 3T3 cells. The bottom row of spots represents the unreacted chloramphenicol. The upper two spots are the acetylated derivatives of chloramphenicol. The assay shown is experiment C from Table 1. Lanes: 1, pSV2cat; 2, BSDcat; 3, SV40-BSDcat; 4, Mo-MuLV-BSDcat.

enhancer was tested, the specificity was more pronounced. The Mo-MuLV enhancer is able to increase expression from the Afp promoter in BWTG3 cells but not in C127 or NIH 3T3 cells (Fig. 1, lanes 4; Table 1). The inability of the Mo-MuLV enhancer to function in the NIH 3T3 cells is not an inherent

Table 1. Expression of CAT activity from the Afp promoter

| Cell type | Exp. | BSDcat | SV40-BSDcat | Mo-MuLV-BSDcat |
|---|---|---|---|---|
| BWTG3 | A | 3.1 | 50 | |
| | B | | 49 | 31 |
| | C | 4.2 | 245 | 15 |
| | D | 2.7 | 22 | |
| C127 | A | 2.2 | 18 | |
| | B | | 16 | 0.7 |
| | C | 2.0 | 83 | 1.3 |
| | D | 0.7 | 7.4 | |
| NIH 3T3 | A | 2.3 | 49 | |
| | B | | 39 | 2.9 |
| | C | 1.7 | 31 | 2.0 |
| | D | 0.6 | 10 | |

After thin-layer chromatography, the spots corresponding to acetylated chloramphemicol were cut out and counted. The specific activity (pmol acetylated per hour per mg of protein) was determined for each experiment; data are expressed as percentage of pSV2cat activity in each cell type.

property of the enhancer, because it has been shown to activate the thymidine kinase promoter in NIH 3T3 cells (37). Also, we tested the effect of the Mo-MuLV enhancer on the SV40 promoter and found that it is able to increase expression from this promoter in all three cell types (Fig. 2). Therefore, we attribute the tissue specificity of Mo-MuLV-BSDcat expression to the Afp sequences present in this plasmid. Cell specificity is also observed with SV40-BSDcat, but to a lesser extent. The cell-specific expression conferred by the Afp sequences may be due to the Afp promoter or it may be due to other elements upstream from the promoter. (The explanations are considered in further detail in the discussion.)

**Afp cis-Acting Elements Exhibit Tissue-Specific Enhancer Activity.** Enhancers are able to activate transcription when located at the 3′ end of the gene as well as the 5′ end. They are also able to activate transcription when linked to heterologous promoters. We tested the upstream Afp sequences to determine whether they exhibited these characteristics of enhancers. The 5.4-kb *Pvu* II fragment was inserted into the *Pvu* II site of BSDcat, in both orientations, to construct the 5′ flanking vectors Afp-CAT and Inv-Afp-CAT (Fig. 3). and into the plasmid pSV1cat at the 3′ end of the CAT gene (Fig. 4). The plasmid pSV1cat was derived from pSV2cat by deleting the SV40 enhancer while leaving an intact promoter region (33). Expression of pSV1cat is very low unless an enhancer is added to it. The recombinants containing the upstream Afp sequences and the Afp promoter are not expressed in C127 or NIH 3T3 cells, but they are expressed at a high level in BWTG3 cells (Fig. 3, lanes 5–8). The orientation of the upstream sequences has no apparent effect on the expression of the CAT gene. The recombinants containing the upstream Afp sequences and the SV40 promoter are also expressed at high levels in BWTG3 cells, and at low but detectable levels in C127 and NIH 3T3 cells (Fig. 4). Therefore, the upstream Afp sequences exhibit the typical characteristics of enhancers: the ability to enhance transcription in a position- and orientation-independent manner and the ability to enhance transcription from heterologous promoters. The cell specificity of the Afp enhancer is shown by

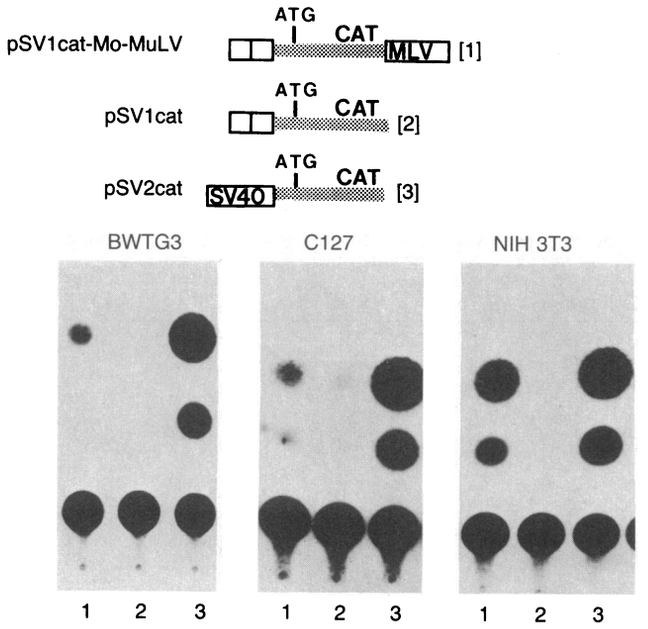


FIG. 2. Activity of the Mo-MuLV enhancer. The Mo-MuLV enhancer was tested in the three cell types for its ability to enhance the expression of pSV1cat. The SV40 promoter is indicated by open boxes. pSV2cat includes the SV40 enhancer also. Lanes: 1, pSV1cat-Mo-MuLV; 2, pSV1cat; 3, pSV2cat.

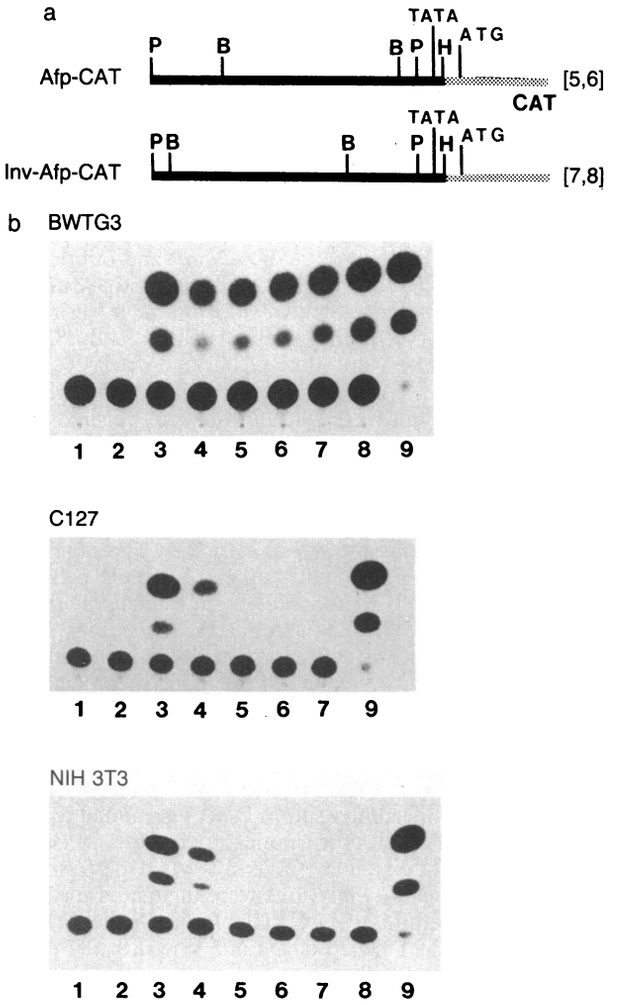


FIG. 3. Addition of upstream Afp sequences to BSDcat. (*A*) The upstream *Pvu* II fragment was inserted, in both orientations, at the *Pvu* II site of BSDcat (see Fig. 1). (*B*) Enhancer activity of the upstream sequences. Lanes: 1, carrier DNA; 2, BSDcat; 3, pSV2cat; 4, SV40-BSDcat; 5 and 6, Afp-CAT; 7 and 8, Inv-Afp-CAT; 9, activity of CAT enzyme purified from *Escherichia coli*. Lanes 5 and 6, 7 and 8 represent separately prepared plasmids. Lane 8 for C127 cells was not done.

the much higher levels of CAT expression in the hepatoma cells, even when the enhancer is used to drive expression from the nonspecific SV40 promoter (Fig. 4).

**Localization of Enhancer Activity by Deletion Mutations.** A series of eight deletions of Afp-CAT was constructed to attempt to localize the enhancer activity in the upstream sequences. These deletions are indicated in Fig. 5 with their levels of expression relative to Afp-CAT. All of the deletion mutants are expressed at lower levels than Afp-CAT, but most retain some enhancer activity. These results indicate that there are probably several enhancer elements present in the upstream sequences. The lower levels of expression of the various mutants indicate that the enhancer activity of the complete fragment may be due to the combined effects of several elements. These elements are located far upstream of the Afp promoter because deletion 3, which retains 2.3 kb of proximal 5′ flanking sequences, completely lacks enhancer activity.

## DISCUSSION

In these studies, we have demonstrated that the 5′ flanking sequences of the mouse *Afp* gene mediate cell-type-specific

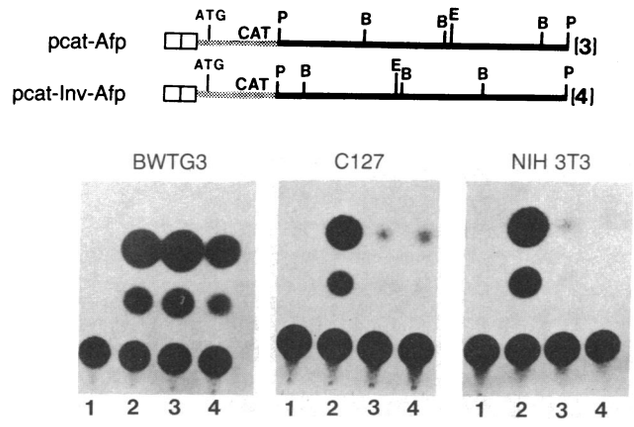


FIG. 4. Analysis of the enhancer activity of the *Pvu* II fragment, using the heterologous SV40 promoter sequences in pSV1cat. (*Upper*) pcat-Afp: The *Pvu* II fragment was placed at the 3′ end of the CAT gene in the 5′→3′ orientation. pcat-Inv-Afp: the *Pvu* II fragment was placed at the 3′ end of the CAT gene in the 3′→5′ orientation. In both cases, the SV40 promoter is at the 5′ end of the CAT gene. (*Lower*) Transient expression assay of the CAT gene in mouse hepatoma (BWTG3), mouse C127 and NIH 3T3 cells. Lanes: 1, pSV1cat; 2, pSV2cat; 3, pcat-Afp; 4, pcat-Inv-Afp.

expression in a transient assay system. Analysis of the regulatory function of these sequences was accomplished by linking them to the bacterial CAT gene. CAT enzyme activity was measured after transfection of Afp-expressing mouse hepatoma cells (BWTG3) and nonexpressing mouse fibroblasts (C127 and NIH 3T3).

We have observed that a fragment containing the Afp promoter and 950 bp of 5′ flanking sequences is able to direct low levels of CAT expression in all three cell types. Expression from the Afp promoter is increased by heterologous enhancers from SV40 and Mo-MuLV. In these experiments, expression of these plasmids relative to pSV2cat expression was consistently higher in the Afp-producing BWTG3 cells. This effect was most apparent with the Mo-MuLV enhancer, which increases transcription from the Afp promoter in the BWTG3 cells but has no effect on the same promoter in fibroblast cells. We showed that the Mo-MuLV enhancer is able to increase transcription from the SV40 promoter in all

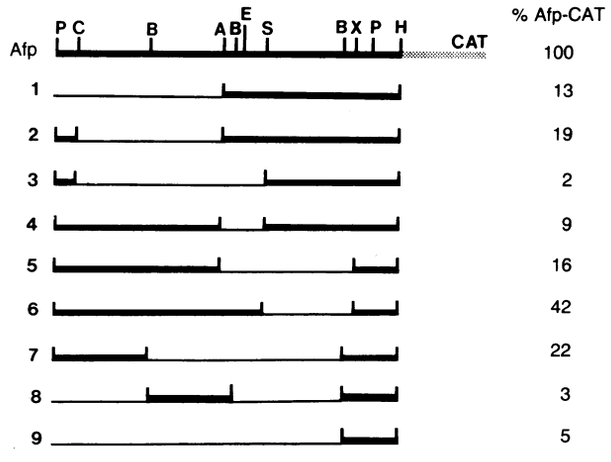


FIG. 5. A deletion analysis of the regulatory activity of 5′ flanking sequences of the mouse *Afp* gene. DNA sequences assayed in the expression vectors are indicated by the heavy black line; deleted regions are indicated by the thin line. P, *Pvu* II; C, *Cla* I; B, *Bam*HI; A, *Ava* I; X, *Xba* I; E, *Eco*RI; S, *Sst* I. Values on right represent percentage Afp-CAT expression and are the average of three independent experiments.

three cell types. Therefore, we conclude that the higher level of expression in BWTG3 cells is due to the Afp sequences. The differences we observed between the levels of CAT expression from Mo-MuLV-BSDcat and SV40-BSDcat are probably due to intrinsic differences in the enhancers, and it appears from our studies that the SV40 enhancer is stronger than the Mo-MuLV enhancer in the cell types that we used.

It has recently been shown that sequences upstream of the Afp TATAA box, from −85 to −52, are required for transcription in hepatoma cells (30) but not in HeLa cells (32). It is likely that the cell specificity we observe is due to these sequences. Our results show that these sequences confer tissue specificity even when under the influence of nonspecific viral enhancers. Sequences with similar properties have been identified in the promoter regions of the rat insulin gene (24) and a mouse immunoglobulin gene (29), suggesting that this type of element may be a common feature of genes expressed in a tissue-specific manner. It was suggested that these sequences may interact with trans-acting molecules that control the expression of the genes (24, 29, 30).

The 5.4-kb *Pvu* II fragment containing sequences upstream from the Afp promoter exhibits the typical characteristics of an enhancer. It increases transcription from the homologous Afp promoter and the heterologous SV40 promoter in a position- and orientation-independent manner. When the Afp enhancer and promoter are combined, expression is strictly limited to the hepatoma cells. When the Afp enhancer and SV40 promoter are combined, a low level of expression is observed in the fibroblasts, but a much higher level is found in the hepatoma cells. Therefore, the enhancer activity found in the 5′ flanking region of the *Afp* gene also confers a significant degree of cell specificity. Similar results were obtained by Godbout *et al.* (30) by linking the enhancer sequences to the viral *TK* gene.

Studies by deletion analysis by Godbout *et al.* (30) and in our laboratory have indicated that the enhancer activity is distributed over several kilobases of DNA. In our studies, however, we have observed that the 1.5-kb *Bam*HI fragment, which is 3.8 kb upstream of the *Afp* gene, does not exhibit enhancer activity in mouse hepatoma cells when linked to BSDcat (see deletion 8 in Fig. 5). A similar construction with the 1.5-kb *Bam*HI fragment in the inverted orientation also showed a lack of activity (data not shown). In contrast to our observations, Godbout *et al.* (30) found that this fragment exhibits enhancer activity in both HepG2 and HeLa cells. These differences may be due to the methods used to assay expression or to differences in the cell types used. With the exception of the 1.5-kb *Bam*HI fragment, we are in agreement with the studies of Godbout *et al.* (30) in that the Afp flanking sequences contain (*i*) a proximal element that lacks enhancer activity but confers tissue specificity and (*ii*) a series of enhancer elements that extend up to 7 kb upstream. These enhancers are strongest in cells that express Afp. Although the fragment that we studied clearly showed cell-specific enhancer activity, we cannot rule out the presence of other enhancers. In particular, although we have not yet tested the 1 kb of DNA that is missing from the *Pvu* II fragment, Godbout *et al.* (30) have shown that this region has enhancer activity. However, the studies from both laboratories indicate that this region is not required for the tissue-specific enhancer activity of the regions further upstream.

It has been proposed in several laboratories that the cell-specific activity of flanking DNA sequences may be due to interactions with trans-acting tissue-specific factors (24, 29, 30, 38, 39). Tissue-specific gene activity could, therefore, be determined by the presence or absence of trans-acting factors that interact with the regulatory sequences to activate or repress transcription. The presence of multiple cis-acting elements for a particular gene suggests that several trans-acting factors may be responsible for determining the activity

of the gene. The ability to test the cis-acting elements separately, as demonstrated in this report, will enable us to ask how Afp expression is controlled under certain conditions. For instance, we have recently shown (unpublished data) that Afp-CAT is not expressed efficiently in primary mouse hepatocytes, which repress Afp expression. We can now ask if this type of control is mediated through the enhancers, the proximal element, or through as yet unidentified sequences.

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