Derepression of a mouse α-fetoprotein expression vector in COS-1 cells by amplification of specific *cis*-acting sequences of the AFP promoter

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ABSTRACT

The existence of trans-acting regulatory factors has been demonstrated by *in vivo* competition with cis-acting sequences from both viral and eukaryotic genomes. Plasmids containing a functional SV40 origin of replication when transfected into permissive SV40 T-antigen producing COS-1 cells will amplify to high copy numbers (5,000 to 10,000) without inflicting toxic effects upon the host cell. This amplification vector (pSVori) has been used to amplify cis-acting regulatory elements which can act as competitors for positive and negative trans-acting factors *in vivo*. Using this amplification system we conducted experiments to determine whether amplification of α-fetoprotein (AFP) and albumin cis-acting promoter sequences could activate a corresponding co-transfected AFP-promoter-CAT or Alb-promoter-CAT expression vector in COS-1 cells. We used pMoMLV(−1009)AFPcat, or p(−308)Albcat-MoMLV as reporter genes and pSVori to amplify specific promoter sequences of the AFP or albumin promoter. Our experiments indicated that amplification of a region from −53 to −202 of the AFP promoter resulted in the activation of the pMoMLV(−1009)AFPcat and p(−308)Albcat-MoMLV expression vectors in COS-1 cells. Surprisingly, amplification of the albumin promoter sequences failed to activate either the pMoMLV(−1009)AFPcat or p(−308)Albcat-MoMLV plasmids.

INTRODUCTION

Cis-elements responsible for regulating cell-type specific and developmental stage-specific transcription of the mouse α-fetoprotein (AFP) gene have been identified in both promoter and enhancer regions (1−7). Similar regulatory elements have also been identified in the rat (8,9) and human AFP genes (10). Transient expression analyses with plasmids containing the mouse AFP promoter and heterologous enhancers have shown that this promoter is capable of directing cell-specific transcription (1,2,4). Such expression vectors direct high levels of expression in hepatoma cells, but are not expressed in fibroblasts. These studies indicate that elements within the promoter play a role in AFP regulation in both AFP expressing and non-expressing cell types. Several hypotheses to explain the absense of expression in non-hepatocyte cell types have been proposed. One proposal is that the concentration of specific positive transcription factors in different cell types is limiting and that the abundance of these factors controls the transcription of genes. A second hypothesis is that transcription may be repressed by specific diffusible regulatory molecules that bind to DNA cis-elements, or to positive transcription factors.

If failure of liver-specific gene expression in fibroblasts (or non-hepatic cell types) involves interaction of trans-acting negative regulatory factors with cis-acting DNA elements, it should be possible to activate these genes by interfering with this regulatory interaction. For example, introduction of a large number of copies of cis-acting elements into the non-expressing cell should compete for the available negative trans-acting factors and result in activation of the silent target gene.

Experiments designed to demonstrate the existence of trans-acting regulatory factors by competition with cis-acting sequences have been reported using both viral (11−13) and eukaryotic (14−16) control elements. SV40 enhancer mediated transcription can be repressed (11,13) and early repression of the SV40 late promoter can be reversed (12) by co-transfection with competitor plasmids. Mercola *et al.*, (14) have reported similar experiments showing repression of immunoglobulin heavy chain enhancer mediated transcription in plasmacytoma cells. Micro-injection of competing mouse β-major globin upstream flanking sequences has been shown to inhibit expression of a β-major globin-TK reporter plasmid (15).

Recent studies have shown that plasmids containing a functional SV40 origin of replication, when transfected into permissive SV40 T-antigen producing cells, will amplify to high copy numbers (5,000 to 10,000), without inflicting toxic effects

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(16,17). Nir *et al.*, (16) used this system to amplify DNA sequences in T-antigen producing COS-1 cells. In these experiments amplified cis-acting regulatory elements act as competitors for the binding of negative regulatory factors of the rat insulin gene, thus allowing activation of the insulin promoter in non-insulin producing cells (16). Furthermore these extrachromosomal DNA-protein complexes were isolated and shown to bind specific potential trans-acting factors (17).

Using this amplification system, we designed experiments to determine whether amplification of AFP or albumin cis-acting promoter sequences could activate the expression of co-transfected AFP promoter-CAT or albumin promoter-CAT expression vectors in COS-1 cells. We used pMoMLV(−1009)AFPcat and p(−308)Albcat-MoMLV, which contain the Moloney murine leukemia virus (MoMLV) enhancer and the AFP or albumin promoters which were fused to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene (2,4). These expression vectors were used as markers for activation of the AFP or albumin promoters in competition assays. To amplify promoter sequences in COS-1 cells, we constructed the pSVori amplification vector. Fragments of the AFP or albumin promoters were inserted into pSVori, and both amplification and reporter plasmids were co-transfected into COS-1 cells to determine if these promoter sequences will activate the expression vectors i.e., pMoMLV (−1009)AFPcat or p(−308)Albcat-MoMLV. Furthermore, by using discrete fragments of the AFP or albumin promoters inserted into pSVori, we attempted to localize regions that are responsible for the failure of these vectors to be expressed in COS-1 cells.

MATERIALS AND METHODS

Plasmids

Several chloramphenicol acetyltransferase (CAT) containing reporter plasmids were used in these experiments. The reporter plasmid, pMoMLV(−1009)AFPcat, consists of the Moloney murine leukemia virus enhancer linked to the −1009 to +37 AFP promoter fragment (Fig. 1B) fused to the bacterial CAT gene (2,4). The p(−6983)AFPcat plasmid includes the −6983 to +37 bp 5′ segment of AFP fused to CAT and contains AFP enhancer and promoter sequences. An additional reporter plasmid p(−308)Albcat-MoMLV is formed by fusing the −308 to +12 albumin promoter to CAT driven by the MoMLV enhancer inserted 3′ to CAT (18).

The prolactin-CAT reporter plasmid pP51 (19) was a gift of Dr. Scott Supowit. The plasmid pSV1catMoMLV was included as a positive control and contains the SV40 early promoter and the Moloney murine leukemia virus enhancer.

The amplification plasmid pSVori was constructed from the EcoR1 to HindIII fragment of pSV2neo by inserting the multiple cloning site derived from plasmid pT7/T3−18. An additional amplification plasmid pSVori(en$^-$) was constructed by deleting an SphI to AccI fragment which eliminates the SV40 enhancer but leaves the origin of replication intact.

The plasmid pCH111 was constructed from the bacterial β-galactosidase gene containing plasmid pCH110 (Pharmacia LKB Biotechnology, Piscataway, N.J.) by replacing the SV40 promoter and enhancer (including origin of replication) with the early promoter and enhancer from polyoma virus. This plasmid was used to control for transfection efficiency (20).

The AFP parent competitor plasmid pSVori(−839/+56)AFP was constructed by inserting the XbaI (−839) to Sau3A (+56) AFP 5′ fragment into the multiple cloning site of pSVori (Fig. 1A). This large competitor fragment was further divided using two techniques. First, the XbaI to Sau3A fragment was cut into two fragments using HincII cutting at −53 and inserting the resulting fragments into the multiple cloning site of pSVori (Fig. 1B). Additional deletions were produced by Bal31 digestion from the HincII site (−53), blunt ending, and cloning the deleted fragments into the HindIII and SmaI sites of pSVori. All end point deletions were mapped by cloning into M13mp19 and sequencing. An additional competitor plasmid was constructed by inserting the −308 to +12 albumin promoter fragment into the multiple cloning site of pSVori to produce pSVori(−308/+12)Alb.

Transfections

Transfections were performed according to published techniques (2,21). COS-1 cells which were maintained in culture as described by Gluzman (22), were plated at a density of 0.5×10^6 cells per 100 mm culture dish. The DNA precipitate was prepared by using 5 μg of each plasmid/dish plus sheared salmon sperm DNA to a final concentration of 30 μg DNA/ml. Cells were harvested by scraping and resuspended in 100 μl of 0.25 M Tris-HCl, pH 8.0, 0.5 mM dithiothreitol. Extracts for enzyme assays were prepared by 4 freeze-thaw cycles and centrifugation to remove cell debris. Protein concentration was determined by the technique of Bradford (23) and 30 μg of protein were used for each enzyme determination. The β-galactosidase activity was determined as described by Miller (24). CAT enzyme activity was determined as described previously (25). The relative efficiency of

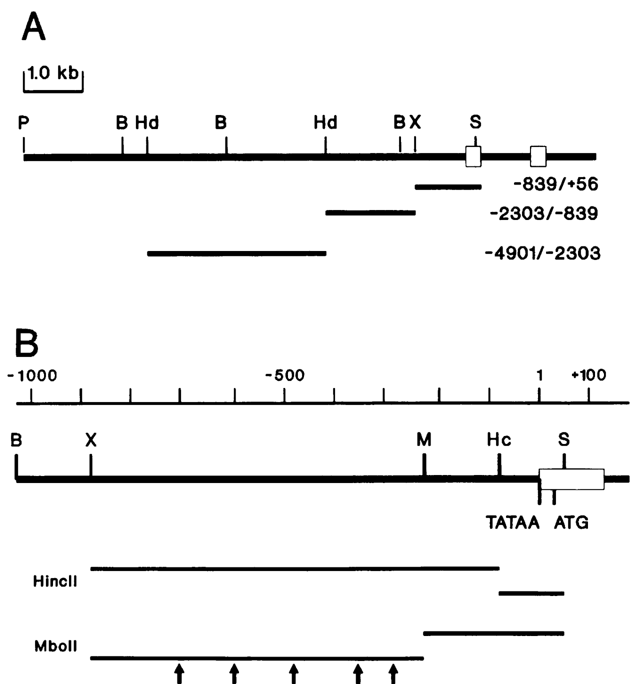

Figure 1. (A) Genomic map of the mouse AFP upstream region showing the location of cloned fragments (−839/+56)AFP, (−2303/−839)AFP, and (−4901/−2303)AFP. (B) Map of the AFP promoter showing upstream flanking region up to nucleotide −1009 in the BamH I site and fragments used for competition assays. Arrows show endpoints of Bal31 generated deletions. Letters C-I identify fragments used in Figure 3. Open boxes denote the location of AFP exons 1 and 2. Restriction enzymes: B, BamH I; Hc, HincII; Hd, HindIII; M, MboII; P, PvuII; S, Sau3A; X, XbaI.

transcription of the reporter gene was expressed as a ratio of the total amount of [^{14}C]chloramphenicol/μg protein/hour and normalized to β-galactosidase activity.

RESULTS

Hepatocyte specific expression of the pMoMLV(−1009)AFPcat and p(−6983)AFPcat vectors has been attributed to cis-acting sequences of the AFP promoter domain (7). Therefore, failure of non-hepatocyte cells, such as fibroblasts, to support CAT expression directed by these plasmids could be attributed to regulatory events mediated by cis-acting sequences within the promoter. In order to examine this question, using SV40 derived amplification vector competition, we first needed to demonstrate that p(−6983)AFPcat and pMoMLV(−1009)AFPcat were not expressed when transfected into COS-1 cells.

The data in Figure 2A (lane 4) and Figure 2B (lane 1) show very low levels of CAT enzyme activity directed by p(−6983)AFPcat and pMoMLV(−1009)AFPcat respectively, indicating that these plasmids are not expressed in COS-1 cells. Furthermore, the absence of CAT activity cannot be attributed to the heterologous MoMLV enhancer used in the constructions, as the plasmid pSV1catMoMLV is active in COS-1 cells (Fig. 2B, lane 5) whereas p(−6983)AFPcat which includes the AFP enhancer domains is not (Fig. 2A, lane 4).

As a first step toward identifying such cis-acting sequences, pSVori (−839/+56)AFP, pSVori(−2303/−839)AFP, and pSVori(−4901/−2303)AFP, each of which includes a different genomic fragment from the 5′ region of the AFP gene (Fig. 1A), were co-transfected into COS-1 cells along with either p(−6983)AFPcat or pMoMLV(−1009)AFPcat reporter vectors. The data indicate that CAT activity from both reporters is greatly increased in co-transfected cells only with the competitive plasmid pSVori(−839/+56)AFP (Fig. 2A, lane 3 and Fig. 2B, lane 2). The pSVori vector alone causes a slight increase in activity (Fig. 2A, lane 1 and Fig. 2B, lane 4), although much less than with pSVori(−839/+56)AFP. In addition, neither pSVori(−4901/−2303)AFP (Fig. 2A, lane 2) nor pSVori(−2303/−839)AFP (Fig. 2B, lane 3) significantly increase CAT activity above the levels seen with pSVori alone. Therefore, we interpret these data to mean that the presence of multiple copies of the AFP promoter sequence [pSVori(−839/+56)AFP] may compete for the binding of negative regulatory trans-acting factors allowing the reporter genes to be expressed. For further analysis we chose to use pMoMLV(−1009)AFPcat as the reporter plasmid and a series of deletions of pSVori(−839/+56)AFP as competitors.

To localize potential negative regulatory cis-elements within the AFP promoter, we constructed a panel of competitor plasmids containing different deletions of pSVori(−839/+56)AFP (Fig.

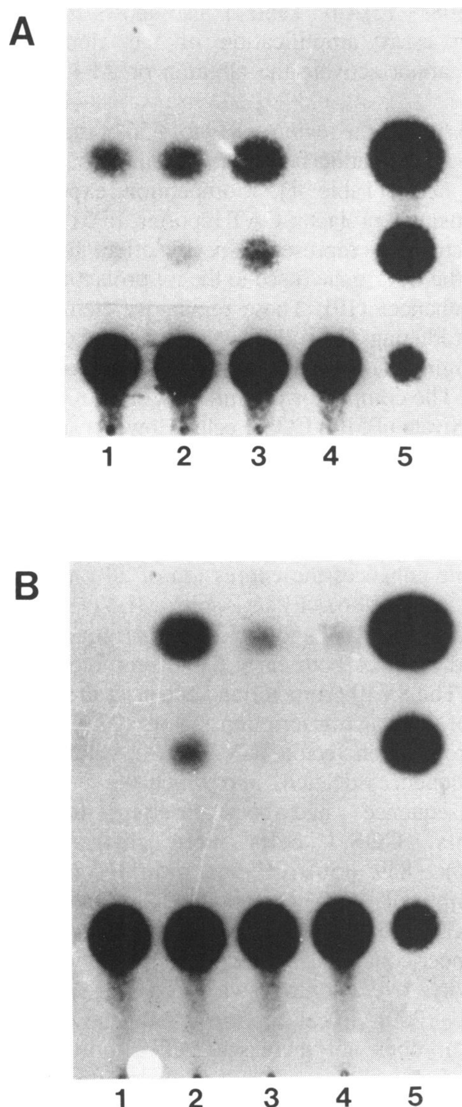

Figure 2. Transient expression analysis from an *in vivo* DNA sequence competition experiment in which AFP promoter sequences were amplified. Transfections and assays were performed as described in *Materials and Methods.* Panel A shows results using p(−6983)AFPcat and Panel B shows results using pMoMLV(−1009)AFPcat as the reporter genes. (A) Lane 1: pSVori + p(−6983)AFPcat; Lane 2: pSVori(−4901/ −2303)AFP + p(−6983)AFPcat; Lane 3: pSVori(−839/+56)AFP + p(−6983)AFPcat; Lane 4: p(−6983)AFPcat; Lane 5: pSV2cat. (B) Lane 1: pMoMLV(−1009)AFPcat; Lane 2: pMoMLV(−1009)AFPcat + pSVori(−839/+56)AFP; Lane 3: pMoMLV(−1009)AFPcat + pSVori(−2303/−839)AFP; Lane 4: pMoMLV(−1009)AFPcat + pSVori; Lane 5: pSV1catMoMLV.

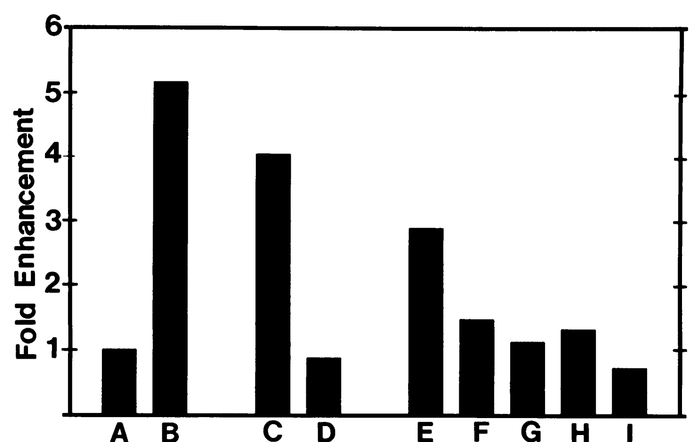

Figure 3. Ability of deletions of the AFP promoter sequence to derepress pMoMLV (−1009)AFPcat. Transfections and CAT assays were performed as described. Values have been corrected for transfection efficiency and normalized to activity in pSVori + pMoMLV(−1009)AFPcat control cotransfected cells. Results shown are averages of 3 experiments. The fragments used as competitors are as follows: (A) pSVori alone; (B) −839 to +56. (C) −839 to −53. (D) −53 to +56. (E) −202 to +56. (F) −839 to -289. (G) −839 to −360. (H) −839 to −600. (I) −839 to −700. End points are numbered with respect to the transcription initiation site.

Table 1. Activation of the albumin promoter-CAT vector. COS-1 cells were transfected with the expression vectors AFP-CAT [pMoMLV(−1009)AFPcat] or Albumin-CAT [p(−309)AlbCatMoMLV] and co-transfected with the competitors pSVori, pSVori (−839/+56)AFP or pSVori(−309/+12)Alb. The data from three experiments is presented as nM chloramphenicol acetylated/mg protein/hr. (Mean ± SD.)

COMPETITOR	REPORTER	
	pMoMLV(-1009)AFPcat	p(-309)AlbCatMoMLV
NONE	0.069 +/-.031	0.049 +/-.036
pSVori	0.143 +/-.042	0.092 +/-.046
pSVori(-839/+56)AFP	1.249 +/-.269	0.438 +/-.017
pSVori(-309/+12)Alb	0.167 +/-.044	0.117 +/-.064

Table 2. Activation of the prolactin-CAT vector. COS-1 cells were transfected with AFP-CAT [pMoMLV(−1009)AFPcat] or prolactin-CAT [pP51] reporters and co-transfected with either pSVori or pSVori(−839/+56)AFP as competitors. The data from three experiments is presented as nM chloramphenicol acetylated/mg protein/hr. (Mean ± SD.)

REPORTER	COMPETITOR		
	NONE	pSVori	pSVori(-839/+56)AFP
pMoMLV(-1009)AFPcat	0.124 +/-.023	0.529 +/-.048	2.65 +/-.156
pP51 [Prolactin-CAT]	0.629 +/-.076	2.41 +/-.324	2.96 +/-.421

Table 3. Effect of SV40 enhancer sequences on activation by pSVori replication plasmids. COS-1 cells were transfected with AFP-CAT [pMoMLV(−1009)AFPcat] reporter plasmid and co-transfected with replication vectors pSVori or pSVori(en⁻), or AFP promoter sequence containing derivatives pSVori(−839/+56)AFP or pSVori(en⁻) (−839/+56)AFP. The data from three experiments are presented as nM chloramphenicol acetylated/mg protein/hr. (Mean ± SD.)

COMPETITOR	REPORTER pMoMLV(-1009)AFP
NONE	0.118 +/-.015
pSVori	0.637 +/-.173
pSVori(-839/+56)AFP	2.363 +/-.338
pSVori(en-)	0.136 +/-.036
pSVori(en-)(-839/+56)AFP	0.424 +/-.070

1B). Each of these competitor plasmids was cotransfected with pMoMLV(−1009)AFPcat and their relative abilities to activate the expression vectors are presented in Figure 3. These fragments fall into two categories, those that can activate the reporter plasmid, and those that have no effect. The entire promoter region in pSVori(−839/+56)AFP gives the most effective activation (Fig. 3B). Two other fragments, spanning −202 to +56 (Fig. 3E) and −839 to −53 (Fig. 3C) respectively, are also able to activate pMoMLV(−1009)AFPcat. The common sequence in these overlapping clones is between −202 and −53. In addition, the fragment spanning −53 to +56 (Fig 3D) has no effect. Therefore, these data suggest that a sequence element in the region between −202 and −53 of the AFP promoter is important for activation of pMoMLV(−1009)AFPcat in COS-1 cells using this competition assay.

Scott and Tilghman (26) have reported two blocks of sequence homology between mouse AFP and albumin located within the first 100 nucleotides upstream of both genes, while the remainder of the 5′ flanking regions show little homology. Therefore, we investigated the ability of pSVori(−839/+56)AFP to activate an albumin promoter-CAT reporter plasmid. The albumin promoter plasmid p(−308)AlbcatMoMLV, which has been shown to be expressed in a tissue specific manner (18), is not expressed in COS-1 cells (data not shown). AFP competitor plasmid pSVori(−839/+56)AFP was co-transfected with the albumin reporter p(−308)AlbcatMoMLV into COS-1 cells. The results are shown in Table 1. The AFP promoter fragment was able to enhance CAT expression by slightly more than 3 fold. In addition the ability of albumin upstream sequences to activate the albumin promoter was examined using the albumin competitor plasmid pSVori(−308/+12)Alb. Table 1 also shows that in a similar competition assay amplification of the albumin promoter sequences cannot activate the albumin or AFP tissue specific promoters.

Sequences within the region −839 to +56 of the AFP promoter are capable of activating both the AFP and albumin promoters in COS-1 cells (Table 1). Competition experiments were performed using a prolactin-CAT reporter, pP51 to demonstrate that these activations represent a specific effect. The vector pP51 consists of the CAT gene fused to the rat prolactin promoter and proximal enhancer (19). These regulatory elements are tissue specific, producing CAT expression only in certain cells of pituitary origin (27). The results of these experiments are shown in Table 2. The competitor plasmid pSVori(−839/+56)AFP is unable to activate pP51 in COS-1 cells. However, the non-specific enhancing effect of pSVori alone on CAT expression noted earlier (Fig. 2A, lane 1; Fig. 2B, lane 4) is observed here with both pP51 and pMoMLV (−1009)AFPcat reporters.

In the experiments described here, the competitor plasmid pSVori alone enhances the expression of all reporter plasmids examined [p(−6986)AFPcat, pMoMLV(−1009)AFPcat, p(−308)AlbcatMoMLV and pP51]. The origin of replication in this plasmid includes both early SV40 promoter and enhancer sequences. The SV40 promoter and enhancer are known to bind a number of cellular transcription factors (27−31). Therefore, we used a replication vector, pSVori(en⁻), which has the SV40 enhancer sequences deleted, into which the (−839/+56)AFP promoter sequences had been inserted, for competition experiments. COS-1 cells were co-transfected with pSVori(en⁻)(−839/+56)AFP and pMoMLV(−1009)AFPcat and compared to control transfections using pSVori(−839/+56)AFP. The results are shown in Table 3. The SV40 enhancer containing vector, pSVori, shows an increase in CAT activity over control when co-transfected with the pMoMLV(−1009)AFPcat reporter while the deleted vector, pSVori(en⁻), does not increase CAT activity over control. These two replicating vectors, with inserted (−839/+56) promoter sequences, were compared for their ability to activate pMoMLV(−1009)AFPcat. Both pSVori(−839/+56)AFP and pSVori(en⁻)(−839/+56)AFP were able to activate the expression vector to approximately the same extent (3.7 and 3.1 fold respectively) indicating that the SV40 enhancer sequences contribute to a background level of enhanced expression by the reporter plasmid, but does not contribute to the −839 to +56 sequence specific activation.

DISCUSSION

In these studies, we used an *in vivo* competition assay to localize the cis-elements of the AFP promoter involved in the negative

regulation of the AFP gene in non-hepatic cell types. These studies indicate that a region of the AFP promoter from −202 to −53 may contain sequences responsible for this negative regulation of AFP in COS-1 cells. Tilghman *et al.*, (1) have reported deletion studies that implicate the region between −85 and −52 of the mouse AFP promoter in tissue-specific expression. These reports are consistent with the results presented here.

Analysis of liver-specific gene extinction in somatic cell hybrids has provided evidence that AFP expression is under the control of trans-acting factors *in vivo*. It has been shown that in hepatoma×fibroblast hybrid cells, liver-specific genes, including AFP, are extinguished (32−34). Albumin expression is also extinguished in hepatoma×enucleated fibroblast cybrids (35) indicating a diffusible factor is involved in the mechanism. This is also supported by the observation that extinction has been shown to reside on a single fibroblast chromosome for albumin (36) and tyrosine aminotransferase (37) and that this locus is not linked to these target genes. These findings provide strong evidence for the presence of negative trans-acting factors in fibroblasts. Such dominant trans-acting negative regulatory loci have been termed *Tse* loci by Killary and Fournier (37). Experiments using AFP promoter-CAT transient expression vectors in hybrid cell lines suggest that these trans-acting factors may either interact with sequences located within 1 kb upstream of AFP *i.e.*, the (−839/+56) fragment or may abolish the activity or production of trans-acting factors that bind to the AFP promoter (4). If the mechanism involves the interaction of a negative protein factor with promoter sequences then activation of extinguished genes such as AFP or albumin should occur by amplification of specific promoter sequences. We observed activation of an AFP expression vector by amplification of very specific promoter sequences, and we believe these studies localize sequences essential to AFP regulation in non-expressing cells.

Zhang *et al.*, (38) have demonstrated that there are 14 protein binding sites in the AFP promoter region (−839 to +56), using nuclear proteins from the adult liver. Sequence analysis of the proximal promoter region, up to −202 bp has revealed that there are 5 binding regions which include sites for liver specific (C/EBP and HNF-1) and ubiquitous (NF-1) trans-acting factors. Binding sites for two nuclear proteins, NP-III and NP-IV which may be associated with the repression of the AFP gene in the adult liver

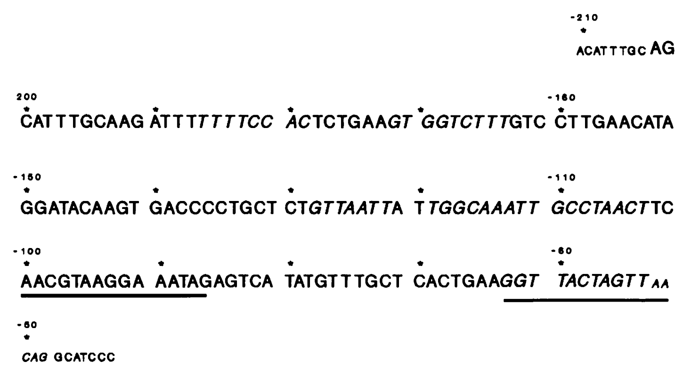

Figure 4. Sequence of the proximal promoter region of the mouse AFP gene. The sequence from −202 to −53 is shown in large type. The concensus binding sequences for trans-acting factors discussed in the text are shown in italics. The underline marks the region of homology between the mouse albumin and AFP promoters.

are also identified in these studies. The binding sites are aligned in the following order from −1 to −202 (see ref 38 and Fig. 4): HNF-1 (−43 to −62); C/EBP (−62 to −82); NF-1 (−98 to −126); NP-III (−131 to −146); NP-IV (−152 to −170); and C/EBP(−178 to −202). The observed activation of the AFP promoter mediated by amplification of AFP 5′ flanking sequences may be due to competition for trans-acting factors which bind to these consensus sequences. For example, NP-III and NP-IV, which are not present in fetal liver nuclear proteins appear at birth when AFP transcription is being repressed (38). Furthermore, both kidney and brain nuclear extracts contain proteins that bind to the NP-III and NP-IV binding sites of the AFP promoter. The presence of NP-III and NP-IV in kidney nuclear protein suggests that they may be present in COS-1 cells which are of kidney origin. Thus, NP-III and NP-IV may be involved in the repression of the AFP gene and binding of these proteins by amplification of their binding sites may be a part of the mechanism that activates the AFP expression vector.

There are other significant differences in DNase I protection patterns between non-hepatocyte and hepatocyte nuclear proteins that may affect AFP regulation. For example, variant forms of HNF-1 (vHNF-1) have been described which retain similar binding characteristics. However, while HNF-1 is liver-specific, vHNF-1 is found in non-expressing cells such as dedifferentiated hepatoma cells, somatic cell hybrids, and normal non-hepatic tissue (38,40). When these hepatoma cells or somatic cell hybrids are selected for re-expression of liver genes, the return of liver-specific expression is accompanied by the replacement of vHNF-1 by HNF-1 (40). These properties are consistent with those expected for the product of the *Tse* locus. If the binding of such an inhibitory vHNF-1 were responsible for repression in COS-1 cells, the observed activation could result from the amplified promoter sequences competing for binding of this negative regulator.

Our previous studies have also shown that protection of the NF-1 binding site varies between liver and kidney proteins indicating that the kidney nuclear extract contains a different member of the proposed NF-1 multi-gene family (41). Thus in the tissues (adult liver, kidney and brain) where the AFP gene is repressed, there are significant changes in the DNase I protection patterns that may affect HNF-1, NF-1, NP-III and NP-IV interactions.

The rat insulin 1 gene promoter (16) and the mouse AFP gene promoter (data not shown) are not activated in COS-1 cells by co-transfecting with a non-replicating plasmid. This suggests that either replication or some property of an SV40 origin containing plasmid may contribute to the observed competition for these negative regulatory factors. For example, the replication of the SV40 competitor may stabilize a chromatin-like complex which might irreversibly bind negative regulatory factors.

Wang and Calame (13) have shown that the SV40 enhancer forms a stable protein complex which becomes resistant to inhibition by competing sequences after transcription has begun. If such a complex were to include an AFP negative regulatory molecule it might be irreversibly bound in this amplification system. A recent paper also suggests that extinction mediated by the mouse IgH enhancer and promoter may involve an interaction between negative regulatory factor and the transcription complex rather than simple binding to a DNA sequence to exert its effect (42). The −839 to +56 AFP fragment contains the functional AFP promoter and potentially could form such a negative regulatory factor:transcription complex. In the fragment −839 to −53, however, although the basal promoter

region is deleted, the fragment retains the ability to activate. Both replication vectors pSVori and pSVori(en-) contain the functional SV40 early promoter and potentially could produce an interaction between a negative regulatory factor bound to the AFP promoter sequences and a transcription complex formed on the SV40 promoter.

The competition experiments presented here demonstrate activation by amplification of specific AFP promoter sequences. However, these sequences also include binding sites for transcription activating factors such as C/EBP, HNF-1, and NF-1. It is certainly possible that some of these specific positive regulators could also be competed by the amplified sequences. Derepression rather than depletion of positive regulators could result if the negative factors are more limiting in the COS-1 cells than positive factors. It may also be that some positive factors are competed but because AFP can bind a number of positive transcription factors, the loss of a subset of these regulators cannot abolish expression.

Our results indicate that 5′ flanking sequences of the AFP gene are able to activate both AFP and albumin promoters, while the amplified albumin sequences are unable to activate the albumin-CAT reporter plasmid. AFP might be expected to activate albumin as there is a region of sequence homology between albumin and AFP in the −202 to −53 region. However, it is unexpected that albumin sequences would then be unable to compete for negative regulatory factors themselves.

Failure of albumin sequences to activate the expression vectors is difficult to explain. One possible explanation is that the AFP sequences have a greater affinity for the binding of a negative regulatory protein and, therefore, compete more effectively in an amplification assay. Such an effect could be mediated by local interactions among bound proteins affecting affinity or stability of the binding interactions of a negative regulatory factor. Alternatively, one could propose that the albumin sequences might compete more strongly for required positive regulatory factors so that their concentration could become rate limiting during albumin amplification and block any observable activation.

Our studies have shown that in non-hepatic COS-1 cells, the failure of expression of an intact AFP promoter contained in an extrachromosomal vector can be overcome by the amplification of cis-acting DNA sequences. These studies, however, are not capable of demonstrating that the non-expressing state has been reversed. Since the amplification and reporter vectors are co-transfected, the increased copies of promoter sequences may prevent the formation of an inactive complex, as discussed above. It is important therefore, to determine if activation can occur when the amplification is initiated after the reporter vector chromatin structure has been established in the inactive state. Reversal of inactivation could also be tested by determining if the constitutive gene has been activated. This distinction is important, and experiments designed to distinguish between the two possibilities could yield information on the stability of the preformed repressed chromatin complex.

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