

Molecular Analysis of the Distal Enhancer of the Mouse α -Fetoprotein Gene

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The mouse α -fetoprotein (AFP) gene is transcribed at high levels in the visceral endoderm of the yolk sac and fetal liver and at much lower rates in the endoderm of the fetal gut. Expression of the gene *in vivo* requires the presence of at least one of three enhancers which lie in its 5' flanking region. In this report, we establish that the most distal AFP enhancer directed consistent expression of a linked AFP minigene in all three endodermal tissues in transgenic mice. The enhancer is composed of three domains, each of which is essential for full enhancer function by transient transfection assays. DNase I footprinting identified three regions of the enhancer which are protected by human hepatoma nuclear extracts, one of which corresponded to a consensus site for HNF-3 binding. Site-directed mutations in this site caused a 10-fold reduction in enhancer function by transient transfection. In transgenic mice, however, the mutation resulted in sporadic expression of the transgene, dependent on the site of integration. A similar acquisition of position-dependent sporadic expression of the transgene was observed with a mutation in a second protein binding site, despite the fact that this mutation had very little effect on enhancer function as assessed by transient transfection. These studies underscore the value of examining the functions of specific protein binding sites *in vivo*.

The α -fetoprotein (AFP) gene is a useful model for examining the mechanisms by which genes are expressed in a cell-type-specific manner during mammalian development. In the mouse, it is expressed in the visceral endoderm of the yolk sac, fetal liver, and fetal gut, although at very different levels (1, 11, 47, 48). AFP mRNA is most abundant in the yolk sac, representing approximately 10% of total mRNA in that tissue. In the fetal liver it is also abundant, between 1 to 2% of mRNA, whereas the AFP gene is expressed at a much lower level in the fetal gut. After birth, AFP gene transcription rapidly declines in both the liver and gut, such that the concentration of AFP mRNA in the adult liver is less than 0.01% of that in the fetal liver (47). To fully understand the regulation of a gene like the AFP gene, it is necessary to explain not only its restricted tissue distribution but also the factors which determine its very different levels of expression in these cell types.

A number of regulatory elements in the AFP gene have been implicated in its tissue-specific pattern of expression. The activity of the proximal promoter of the gene has been shown to require the binding of the transcription factor HNF-1 in both liver and gut-derived cell lines (13, 48), as well as the binding of one or more members of the C/EBP family (5, 46, 51). In the mouse, three enhancers were identified at positions -2.5 (enhancer I), -5.0 (enhancer II), and -6.5 (enhancer III) kb upstream of the transcription start site. When tested by transfection into tissue culture cells, all of the enhancers exhibited equivalent, and nonadditive, activities in liver-derived cell lines (16, 17). In transgenic mice, however, the enhancers acted additively, in that all three were required to achieve position-independent high-level expression of the gene (20). In addition, each enhancer had a characteristic tissue tropism. That is, enhancer I alone directed expression of a reporter AFP minigene in all three of the appropriate tissues, enhancer II was

active in the yolk sac and liver but not in the gut, and enhancer III's activity was restricted to the yolk sac. These observations led to a suggestion that the levels of expression of the AFP gene were determined by the additive action of the three enhancers, in that the highest-expression tissue, the yolk sac, utilized all three enhancers whereas only one enhancer is active in the gut (20).

This simple model could not accommodate several additional observations. First, enhancer III appeared to be comparable in activity to enhancer I in both liver- and in gut-derived cell lines, contrary to the prediction from studies of transgenic mice that it should be inactive in these cell types (17, 48). Second, enhancer III displayed DNase I hypersensitivity in both fetal and adult liver nuclei, suggesting that it had biological activity in the liver (18). The following study was initiated to reconcile the discrepant activities of the distal enhancer *in vivo* and in transfections and to assess its importance in the overall expression of the AFP gene.

MATERIALS AND METHODS

Cell culture. HepG2 cells were grown in a 1:1 ratio of F12 and Dulbecco's minimal essential medium in the presence of 10% fetal calf serum at 37°C in 5% CO₂. HeLa cells were grown under the same conditions except in Dulbecco's minimal essential medium only.

Quantitation of enhancer activity by chloramphenicol acetyltransferase (CAT) assay. Transient transfections into HepG2 cells were performed by the calcium phosphate method as described previously (16). Forty-eight hours after transfection, cells were harvested, and protein extract was prepared by three successive cycles of freezing in liquid nitrogen and thawing at 37°C. Two microliters of extract was incubated with 20 μ l of 4 mM acetyl coenzyme A, 2 μ l of [¹⁴C]chloramphenicol, and 70 μ l of 1 M Tris-HCl (pH 7.8) in a total volume of 150 μ l at 37°C for 10 to 60 min to ensure that the extent of acetylation was in the linear range. The reactions were stopped by extraction with 500 μ l of ethyl acetate, and the reaction products dried, resuspended in 15 μ l of ethyl acetate, applied to silica plates, and separated by thin-layer chromatography. The plate was exposed to X-ray film at room temperature overnight, and acetylated chloramphenicol was quantitated with a Molecular Dynamics PhosphorImager. These results were normalized to take account of transfection efficiency for each construct by Hirt analysis (21).

Quantitation of enhancer activity by S1 analysis. Forty-eight hours after transfection, the cells were harvested and RNA was isolated (41). A 250-bp fragment spanning the thymidine kinase (*tk*) promoter from the *Bgl*II site at +50

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bp to a *PvuII* site at -200 bp was labeled with [γ - 32 P]dATP by polynucleotide kinase. Twenty-five micrograms of total RNA was added to 50,000 cpm of the probe in 85% formamide-10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.5)-1 mM Na₂EDTA. The samples were heated at 95°C for 5 min, adjusted to 0.5 M NaCl, and incubated at 48°C overnight. The mixture was then digested with S1 nuclease in 3 mM zinc acetate-30 mM sodium acetate (pH 4.5)-0.25 M NaCl at 37°C for 1 h. The reaction was stopped by the addition of EDTA to 10 mM, the mixture was extracted with phenol, and the DNA was precipitated with ethanol and separated on a 7.5% denaturing acrylamide gel. A 60-bp protected fragment, representing *tk* mRNA, was quantitated by densitometric analysis, and the value was normalized for the efficiency of transfection by Hirt analysis.

Constructs. The mutations in the 340-bp *HincII* fragment containing enhancer III was generated by a PCR-based mutagenesis method (26). The following primers were used: for footprint 1 (FP1), 5' CACCTTTATTGACTTTGACATATT 3' (coding), 5' GCAGAGCTACAGCAATGTCAGTGG 3' (noncoding), 5' TTGACCTATATCGATGTCTGGGTC 3' (mutant coding), and 5' GACCTGACATCGATATAGGTCAA 3' (mutant noncoding); for footprint 2 (FP2), 5' CAATTTGTCTCTGATCTCTTTAGTTT 3' (coding), 5' AAATATGTCAAAGTCAATAAAGGTGCA 3' (noncoding), 5' CTGTCTTTTAAGTTCGACAGGACGCTTGGTTGCC 3' (mutant coding), and 5' GGCAACC AAGCTGCCTGTGCAACTTAAAAGGACAG 3' (mutant noncoding); and for footprint 3 (FP3), 5' TTTTAAGTTCGACAGGACGCTTGGTTGCC 3' (coding), 5' ACCCAGACATCGATATAGGTCAAGCAGAG 3' (noncoding), 5' GCACCTTTATTGAAAGCTTCATATTCTGTCC 3' (mutant coding), and 5' GGACAGAAATATGAAGCTTTCAATAAAGGTGC 3' (mutant noncoding). Two hundred fifty nanograms of each primer was added to 2 ng of plasmid DNA in 2.5 mM MgCl₂-10 mM Tris-HCl (pH 8.3)-50 mM KCl-0.2 mM deoxynucleoside triphosphates-5 U of *Taq* polymerase in a total volume of 100 μ l. The DNA was denatured at 94°C for 70 s, prior to 15 cycles at 94°C for 35 s, 48°C for 1 min, and 72°C for 3 min. Finally, the DNA was subjected to one more round of extension at 72°C for 8 min. The DNA was denatured at 100°C for 5 min, gradually cooled to 65°C, and then placed on ice. Ten microliters was transformed into *Escherichia coli* MC1061, and plasmids containing the mutations were identified by the presence of new restriction sites (FP1, *PvuII*; FP2, *HindIII*; and FP3, *DraI*) and confirmed by DNA sequence analysis. The mutants were recloned into the *tk*-CAT vector.

The transgenic constructs were generated by the following cloning steps. For the E construct, a 2.25-kb *SacII*-*BamHI* fragment containing enhancer III was cloned into a vector containing the AFP promoter and minigene (AFP ZE.5; [16]). The C construct was generated by digesting a 2.25-kb *EcoRI*-*BamHI* genomic fragment containing enhancer III with *HincII*. The resulting 963- and 912-bp fragments were isolated, ligated to each other, and cloned into the AFP ZE.5 vector. The E transgenic constructs in which mutations had been introduced into the enhancer were generated by using site-directed mutagenesis followed by cloning into the AFP ZE.5 vector. The same protocol as described above was used except that the 2.25-kb *EcoRI*-*BamHI* fragment was used as a template and the extension cycle at 72°C was for 5 min.

Subclones of enhancer III were generated by digesting the 340-bp *HincII* fragment with either *BamHI*, *BstYI*, or *Sau3A* and cloning the resulting fragments into the *tk* or *tk*-CAT expression vector.

Double-stranded oligonucleotides corresponding to each of the protein binding sites were synthesized with terminal *BamHI* linkers and cloned by blunt-end ligation into the *SmaI* site of pUC19. They were subsequently cloned as *BamHI* fragments into the *tk*-CAT vector in one and four copies.

Generation and analysis of transgenic mice. The DNA constructs were digested with restriction enzymes to separate the mouse DNA from vector sequences. The DNA, isolated by agarose gel electrophoresis and CsCl gradient centrifugation, was injected into (C57BL/6J \times SJL/J) F_2 fertilized eggs (22). The progeny were analyzed for the presence of the transgene by PCR or by hybridizing DNA prepared from tails to AFP-specific probes (20). Male transgenic mice were mated to CD1 females, while female transgenic mice were mated to (C57BL/6J \times SJL/J) F_1 males in order to maintain each line. Pregnant CD1 females were sacrificed at embryonic day 17 (e17), and poly(A)⁺ RNA prepared from tissues (3, 4) was analyzed by Northern (RNA) blotting (45).

DNase I footprint analysis. Nuclear extracts were prepared as described by Feuerman et al. (13) and enriched for DNA-binding proteins by heparin-agarose chromatography. Fragments of enhancer III were labeled with [α - 32 P]dATP by the Klenow fragment of DNA polymerase. The probe (~20,000 cpm) was added to 1 μ g of poly(dI-dC) in 25 mM Tris-HCl (pH 8.0)-6.25 mM MgCl₂-0.05 mM Na₂EDTA-50 mM KCl-0.5 mM dithiothreitol-10% glycerol. This mix was incubated on ice for 20 min in the presence or absence of nuclear extracts from HepG2 or HeLa cells in a final volume of 45 μ l. The reaction mixtures were warmed to 25°C for 2 min, and 5 μ l of DNase I (10 mg/ml) diluted in 0.25 M CaCl₂ was added for 1 min. The digested products were separated on a 6% denaturing polyacrylamide sequencing gel.

Gel mobility shift analysis. Double-stranded oligonucleotides containing the binding sites for FP1 (5' GGATCCCTCTGCTTGACCTATATCGATGTCTGGGTGCACCTTTGGATCC 3'), FP2 (5' GGATCCCTGGGTGCACCTTTATTGACCTTTGACATATTTCTGTCCGGATCC 3'), and FP3 (5' GGATCTCTGCTCTTTAAGTTTCGACAGGACGCTTGGTTGCGGGATCC 3') were end labeled with [α - 32 P]dATP, using the Klenow fragment of DNA polymerase

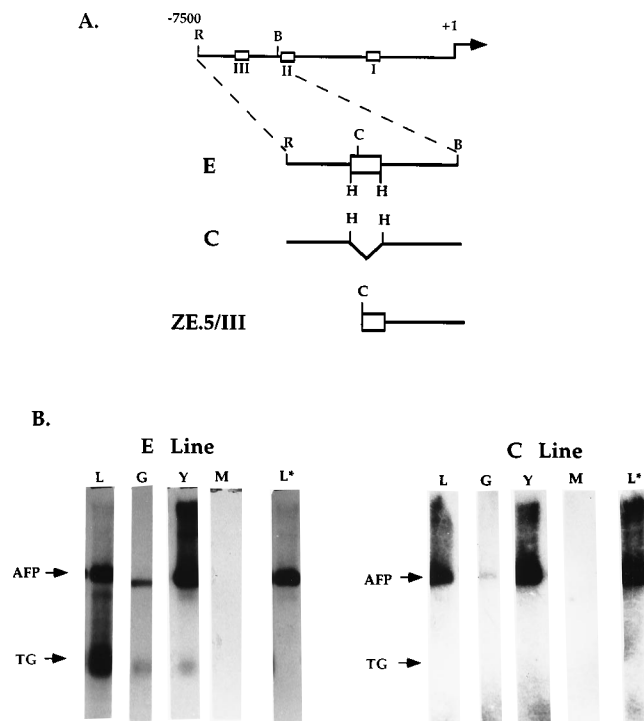


FIG. 1. Enhancer III is required for expression of the AFP minigene in transgenic mice. (A) The 5' flanking region of the murine AFP gene, which includes the three enhancers (open boxes labeled I to III) is depicted, with the arrow representing the start of transcription at bp +1. The portion of the 5' flanking domain included in the E transgene and the sequences deleted in the C and ZE.5/III transgenes are indicated below. R, *EcoRI*; B, *BamHI*; H, *HincII*; C, *ClaI*. (B) RNA from e17 tissues (liver [L], gut [G], yolk sac [Y], and muscle [M]) were separated by agarose gel electrophoresis and hybridized to an AFP-specific probe which recognizes both the endogenous AFP (AFP) and the minigene RNAs (TG). L*, liver from a nontransgenic littermate. The lines shown are E-3 and C-1.

I. Then 0.1 to 1.0 ng (~50,000 cpm) of each probe was incubated with 10 μ g of HepG2 or 5 μ g of Caco-2 cell and tissue nuclear extracts in the presence of 10 μ g each of yeast tRNA and poly(dI-dC) in a final volume of 50 μ l. In some instances, unlabeled competitor oligonucleotides were added at a 100-fold molar excess. The mixtures were incubated for 20 min at 25°C, and the DNA-protein complexes were separated on either 4% native polyacrylamide gels or 1% native agarose gels. The gels were dried and exposed to X-ray film at -80°C.

HNF-3 β RNA was generated *in vitro* by using SP1 RNA polymerase and a linearized plasmid containing HNF-3 β cDNA, a kind gift of E. Lai (30). The RNA was translated in the cell-free reticulocyte translation system (Promega). One-fifth of the reaction mixture was used for each binding reaction.

RESULTS

A reconstituted enhancer III retains activity in liver and gut.

The original transgenic lines that were generated to test the activity of enhancer III utilized an internally deleted AFP reporter gene, containing the first three and last two exons, along with 1 kb of DNA immediately flanking the 5' end of the gene (20). To this was ligated a 1.25-kb *ClaI*-*BamHI* fragment containing enhancer III (AFP ZE.5/III; Fig. 1). In hepatoma cell transfections, this construct exhibited full enhancer III-dependent activity (construct III-A6 in reference 17). Nevertheless, in transgenic mice, AFP minigene expression was consistently observed only in the yolk sac, at levels between 9 and 50% of the endogenous levels (Table 1). In liver, the transgene was expressed at a low level in only one line (20). We considered two possible explanations for this discrepancy. First, because the 5' end of ZE.5/III was within 100 bp of sequences determined by transfection to be required for the activity of

TABLE 1. Summary of expression of the AFP minigene in transgenic mice^a

Construct	Copy no.	Tissue distribution of transgene expression (% endogenous AFP mRNA) ^b			
		Liver	Gut	Yolk sac	Muscle
ZE.5/III	4–100	1/8 (12)	4/8 (15–150)	7/8 (9–52)	ND
E-1	13	62	79	9	0
E-2	5	110	20	44	0
E-3	20	70	44	67	0
E-4	60	86	54	61	0
E-5	12	31	0	73	0
C-1	16	0	0	0	0
C-2	60	0	0	0	0
mFP1-1	12	0	0	0	0
mFP1-2	10	0	0	84	0
mFP1-3	12	44	0	35	0
mFP1-4	6	33	0	28	0
mFP2-1	20	0	61	13	0
mFP2-2	40	151	40	13	0
mFP2-3	3	54	0	0	0
mFP2-4	7	0	0	0	0

^a Data for construct ZE.5/III are taken from reference 20. The range of transgene copy numbers of the eight transgenic lines is indicated, along with the range of expression, relative to that endogenous AFP mRNA (expressed as a percentage within parentheses) for each line which expressed the gene, indicated as a ratio. ND, not determined.

^b A tissue is scored as positive for expression of the transgene if its expression is greater than 5% of that of endogenous AFP mRNA in that tissue.

enhancer III, the transgenes possibly had lost critical sequences during integration into the genome or were susceptible to position effects. Alternatively, there could be functional differences in the requirements for enhancer activity in transient and stable transformations.

To test whether additional sequences could reconstitute full endoderm activity to AFP ZE.5/III, two additional constructs were introduced into transgenic mice. An additional 0.9 kb of DNA upstream of enhancer III was cloned into ZE.5/III, and five transgenic lines were established. Pregnant females were sacrificed at e17, and fetuses were analyzed for endogenous AFP mRNA and AFP minigene expression by using poly(A)⁺ RNA from expressing tissues as well as skeletal muscle, a nonexpressing tissue. Figure 1B shows a Northern (RNA) blot analysis for one representative line, in which the two RNAs could readily be distinguished by virtue of the difference in their sizes. For four of five lines, the transgene was expressed in the three appropriate tissues but not in muscle (Table 1). In a single line, expression was absent in the gut but detectable in the other tissues. Therefore, the addition of 0.9 kb of DNA restored the appropriate tissue specificity of the enhancer predicted from transfection studies. In these and all other transgenic mouse lines described in this work, there was no correlation between the expression level and copy number of the transgenes (Table 1), as had been observed previously (20).

It was formally possible that the restoration of activity occurred by virtue of the inclusion of another enhancer that was distinct from that previously defined by transfection studies. To test this idea, we tested a transgenic construct which contained a 340-bp deletion in the region previously identified as responsible for enhancer III activity (C construct; Fig. 1A). Transgenic lines generated with the C transgene exhibited no expression of the minigene in any tissue examined (Table 1 and Fig. 1B). Thus, if a second enhancer exists in the additional sequences, it has no autonomous activity in the absence of enhancer III. Therefore, we conclude that enhancer III is ca-

pable of directing expression of the AFP gene in the liver, yolk sac, and gut, consistent with results of the transfection experiments.

Deletion analysis of enhancer III. To understand the basis for the endoderm specificity of enhancer III, we constructed a series of deletion mutations that divided the enhancer into three regions, labeled A, B, and C in Fig. 2. These were tested alone and in combination for activity by transient transfection into HepG2 cells, using either the herpes simplex virus *tk* or CAT gene as a reporter. In both instances, the promoter was derived from the *tk* gene. HepG2 cells express high levels of endogenous human AFP and have been shown to support high-level expression of AFP constructs by transfection (16, 17).

The 2.25-kb *EcoRI*-*Bam*HI fragment contained in the E construct (construct 4 in Fig. 2C) exhibited approximately 11-fold enhancer activity by transient transfection. A 340-bp *Hinc*II fragment (referred to as III) exhibited slightly higher levels of enhancement, but all further deletions resulted in substantial or complete loss of enhancer activity (Fig. 2A to C). Only the combination of regions A and C (construct IIIA + C) yielded a residual fourfold enhancer activity. If IIIA + C was duplicated, however, it was possible to restore full enhancer activity. The only other duplication with any activity was IIIA + B, which resulted in five- to sixfold-enhanced activity (Fig. 2C and D). These results indicate that the functional components of enhancer III are distributed throughout the 340-bp region and that the duplication of region A, in the presence of either region B or region C, is sufficient for enhancer function. For this reason, we focused our attention on region A.

Identification of proteins which bind to enhancer III. The modular nature of enhancer III was examined further by identifying the nuclear proteins which interact with it. Nuclear extracts were prepared from HepG2 cells and incubated in the presence of labeled DNAs representing regions A and B (bases 1 to 200 in the *Hinc*II fragment) and subjected to DNase I footprinting (Fig. 3). Four protected regions, FP1 (bases 31 to 55), FP2 (bases 63 to 81), FP3 (bases 87 to 102), and a partially protected region between bases 115 and 125, were identified. The extents of each of the first three footprints were slightly different on the two strands, but in each instance, the core sequences protected on both strands were conserved between the mouse and rat genes (Fig. 3B), suggesting that the sequences were relevant to the expression of the gene.

To ascertain which of these DNA binding activities was specific to the liver, and thereby a potential candidate for a factor responsible for the endoderm specificity of the enhancer activity, footprint analyses were also performed with nuclear extracts from HeLa cells in which enhancer III is inactive. Only one footprint was observed, at bp 115 to 125 of the enhancer. This protected region coincided with the fourth footprint obtained with the HepG2 nuclear extracts. The three HepG2-specific footprints were considered potential candidate binding sites for liver-specific transcription factors responsible for the activity of enhancer III.

Mutational analysis of the protein binding sites in enhancer III. The relative contributions of FP1, FP2, and FP3 to enhancer III activity in HepG2 cells was tested by using site-directed mutagenesis to introduce mutations into the sequences protected in each footprint. The mutations were detected by the introduction of novel restriction enzyme cleavage sites into the mutated sequences and confirmed as the only alteration by DNA sequence analysis (data not shown).

To verify that the mutations had disrupted protein-DNA interactions, oligonucleotides containing each footprint were radiolabeled and incubated in the presence of HepG2 nuclear

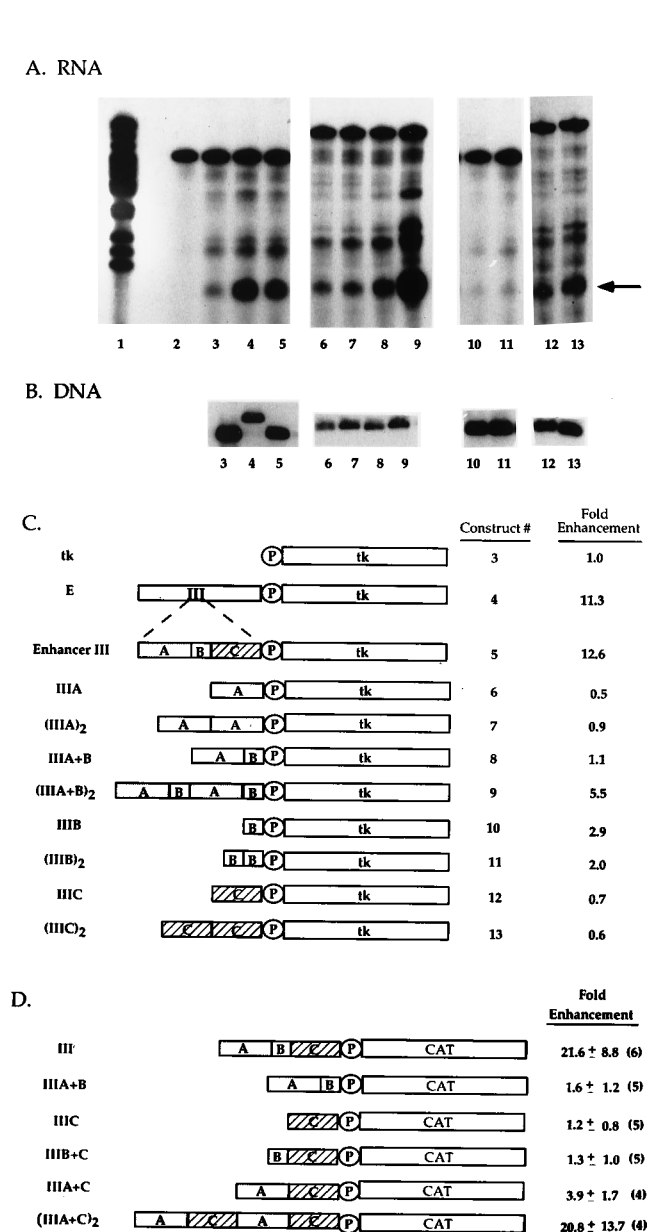


FIG. 2. Molecular dissection of enhancer III. (A) The constructs depicted in panel C were transiently transfected into HepG2 cells, and RNA was prepared 48 h later. The RNA was analyzed by an S1 nuclease protection assay to detect the quantity of *tk* mRNA by the presence of a 60-bp protected band (arrow). Lane 1, radiolabeled pBR322 digested with *MspI*; lane 2, mock-transfected cells. Lanes 3 to 13 refer to the constructs similarly numbered in panel C. (B) Episomal DNA was recovered from the same cell pellets, digested with *EcoRI*, and separated on a 0.8% agarose gel. The DNA was transferred to nitrocellulose and hybridized with a *tk*-specific radiolabeled probe. (C) The reporter *tk* structural gene is indicated by the open box, and its promoter is represented by the open circle. The 2.25-kb E fragment from the AFP gene (construct 4) was subdivided into a 340-bp *HincII* fragment referred to as enhancer III (construct 5). This was further subdivided into fragments A (shaded box; bp 1 to 140), B (open box; bp 141 to 200), and C (hatched box; bp 201 to 340). Combinations of fragments were subcloned upstream of the *tk* reporter gene as indicated. The average fold enhancement of the *tk* reporter gene expression, calculated by densitometry, from three to six separate experiments each is indicated to the right. (D) Additional constructs were also transfected into HepG2 cells by using a CAT reporter gene and the *tk* promoter. The subdivisions of enhancer III are as in panel C. The average fold enhancement \pm standard deviation of the CAT reporter gene is indicated to the right, with the number of times each construct was transfected in parentheses.

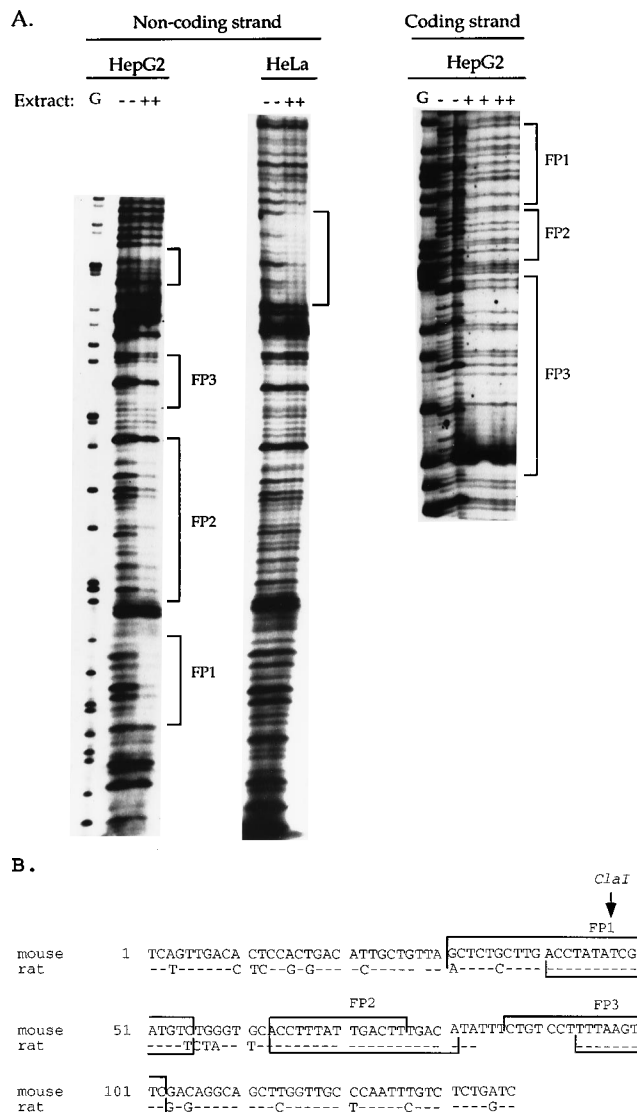


FIG. 3. DNase I footprinting of region A of enhancer III. (A) A fragment encompassing region A of enhancer III was labeled by using the Klenow fragment of DNA polymerase on either the coding or noncoding strand. The fragment was incubated in the absence (–) or presence (+) of duplicate samples of nuclear extracts prepared from HepG2 or HeLa cells, as indicated. For the coding strand, the first two and last four (+) lanes were generated with nuclear extracts representing different fractions eluted from a heparin-agarose column. The mixtures were digested with DNase I and separated on denaturing 6% acrylamide gels. The G lanes represent the same fragments cleaved with dimethyl sulfate and piperidine. (B) The sequence of region A is compared with the same region from the rat gene (6), with dashed lines indicating identity. The extents of the three HepG2-specific footprints are indicated by the boxes. The *ClaI* site, used in the generation of the ZE.5/III transgenic construct, is also indicated.

extracts. As shown in Fig. 4A to C, the protein-DNA complexes, visualized by gel mobility shift analysis, could be disrupted with the corresponding wild-type oligonucleotide but not with oligonucleotides containing each of the mutant binding sites.

These mutant enhancer III constructs were cloned into the *tk*-CAT vector and introduced transiently into HepG2 cells (Fig. 5). Independent of the orientation of the enhancer in the vector, mutations in the FP2 binding site had the most dramatic effect, reducing activity at least 10-fold. Mutation of FP3 decreased enhancer activity less than twofold in either orien-

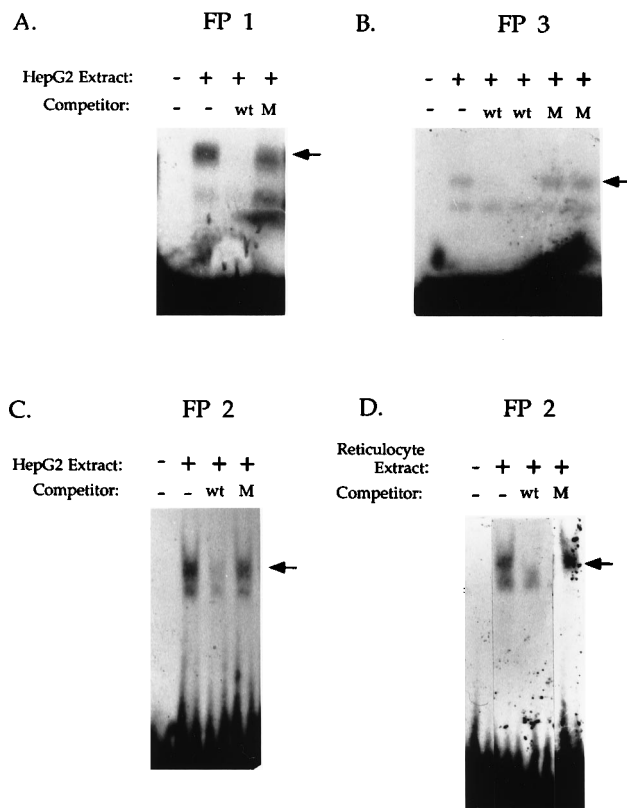


FIG. 4. Effects of site-directed mutations on protein binding to enhancer III. (A to C) Double-stranded oligonucleotides containing the sequences protected from DNase digestion (FP1 to FP3) were radiolabeled and incubated with HepG2 extract in the absence or presence of a 100-fold excess of either a wild type (wt) or mutant (M) oligonucleotide. For FP3, both 100- and 500-fold-excess oligonucleotides were used. The complexes were displayed on a native acrylamide gel. (D) The oligonucleotide containing FP2 was incubated in the presence of a rabbit reticulocyte lysate containing HNF-3 β translated from RNA synthesized in vitro. The arrows indicate the positions of the protein-DNA complexes.

tation, whereas the FP1 mutation in the enhancer cloned in the 5'→3' orientation actually increased enhancer function three-fold but had no effect when the enhancer was cloned in the opposite orientation. This analysis revealed that of the three protein binding sites in region A, the FP2 site is the most critical to the activity of the enhancer by transfection.

The ability of each of the footprints to generate enhancer activity was analyzed further by studying the activities of the sequences in isolation. Each of the sites was cloned in one or four copies into the *tk*-CAT vector. The resulting constructs were introduced transiently into HepG2 cells, and the levels of CAT expression were determined (Fig. 6). Of the six constructs, only the one containing four copies of the FP2 site exhibited enhancer activity, whereas one copy had no effect. In contrast, concatemers of FP1 and FP3 were unable to increase CAT activity. These data confirm the importance of the FP2 site for enhancer III function.

HNF-3 binds to the FP2 site. The regions protected by FP1 and FP3 displayed no apparent sequence similarity to the binding sites for transcription factors which have been characterized to date, including the liver-enriched factors HNF-1 (10, 35, 36), C/EBP (9, 25, 32), and HNF-4 (42). However, the sequences protected by FP2 conform in 13 of 15 bases to the consensus binding site of the mammalian HNF-3 transcription factor family (TTATTGACTTAGTCA). This family of pro-

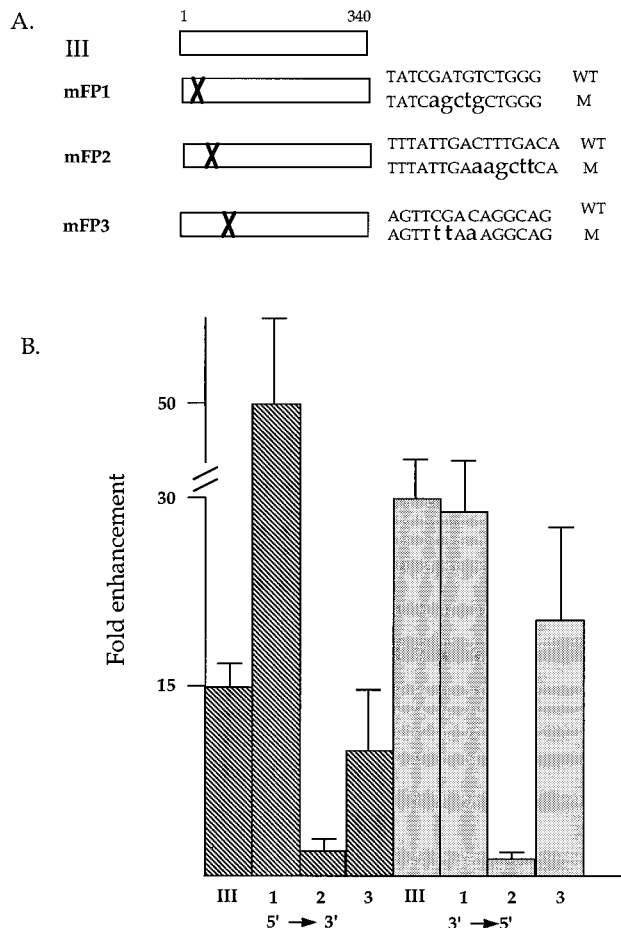


FIG. 5. Effects of site-directed mutations on enhancer activity. (A) The 340-bp enhancer III and each of the site-directed mutations in FP1, FP2, and FP3 binding sites (X's) are indicated. The altered sequences in each mutant (M) are indicated by lowercase letters below the corresponding wild-type (WT) sequence. (B) The wild-type enhancer and each of the mutants (labeled 1 to 3) were cloned in both orientations into the *tk*-CAT vector and introduced transiently into HepG2 cells. The two different orientations are illustrated by different shadings. The average fold enhancement \pm standard deviation for each of the mutants is indicated.

teins, which includes the *Drosophila melanogaster* homolog, fork head (50), were originally identified by virtue of their central role in directing the transcription of the liver-specific genes encoding transthyretin and α 1-antitrypsin (8). Three family members, HNF-3 α , - β , and - γ , have been extensively characterized in mice, and their expression is restricted to endodermal tissues such as the liver, gut, and lung (30, 31).

To investigate whether an HNF-3 family member can bind to FP2, a cDNA clone of HNF-3 β was transcribed in vitro and translated in a rabbit reticulocyte extract. The extract was incubated with a radiolabeled FP2 oligonucleotide, and the mobilities of the complexes formed were compared with those of the complexes obtained with HepG2 extracts. As shown in Fig. 4D, the reticulocyte lysate containing recombinant HNF-3 β generated a pattern of two bands that was very similar to that observed with HepG2 extracts (Fig. 4C). The more slowly migrating band was competed for effectively by excess unlabeled FP2 but not by the mutant oligonucleotide. When excess unlabeled FP1 and FP3 oligonucleotides were used as competitors, the HNF-3 β -specific band was not affected (data not

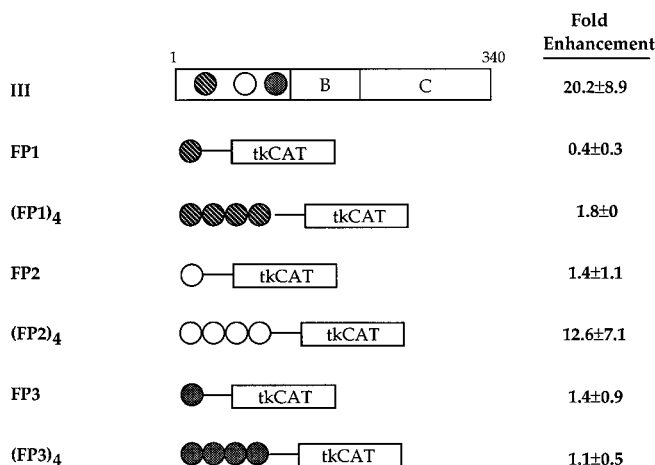


FIG. 6. Enhancer activities of concatemers of DNase I footprints. The positions of the three DNase I footprints in enhancer III are depicted by the hatched (FP1), open (FP2), and shaded (FP3) circles. Each of the binding sites was cloned in one or four copies upstream of *tk*-CAT. These constructs, along with enhancer III, were introduced transiently into HepG2 cells. The average fold enhancements \pm standard deviations of basal *tk*-CAT are indicated.

shown). These results suggest that the band shifting activity in the HepG2 extract is the consequence of an HNF-3 protein.

Analysis of FP1 and FP2 mutations in transgenic mice. The positions of two of the three footprints in enhancer III immediately suggested that their absence (FP1) or possible disruption because of proximity to the *Cla*I site at the 5' end of the construct (FP2/HNF-3) could explain the differences in the biological activities of ZE.5/III and the E construct. To examine these possibilities, the mutations in FP1 and HNF-3 sites described in Fig. 4 were introduced separately into the E construct (Fig. 7), and transgenic mice were generated. The expression of the AFP minigene was examined in F₁ progeny of founders at e17 to e19.

Unlike the E lines themselves, which exhibited significant expression of the transgene in all five founders, each of the four mFP2 lines expressed the transgene differently (Fig. 7 and Table 1). For example, in line mFP2-1, the minigene was transcribed in the fetal gut and yolk sac but not in the liver. However in the mFP2-2 line, minigene transcripts were detected in all three tissues; in mFP2-3, expression was restricted to the liver, and a fourth line failed to express the transgene in any tissue. Despite this variation in the normally expressing tissues, ectopic expression in skeletal muscle was not detected. These results suggest that disruption of HNF-3 binding resulted in a diminution in, rather than an abolition of, the activity of enhancer III, leading to integration-specific position effects of differing severity.

The deleterious effects of the mutation in the HNF-3 binding site on transgene expression were not unexpected, given the fact that the same mutation caused a significant decline in enhancer function in tissue culture cells. In contrast, the modest effect in tissue culture of the mutation in FP1 was not observed when transgenic mice carrying the same mutation were examined for minigene expression (Fig. 7 and Table 1). None of the lines expressed the minigene in the gut, suggesting that FP1 is essential for expression in that tissue. For two of the lines, the minigene mRNA was detected in the fetal liver and yolk sac. For a third, the RNA was restricted to the yolk sac, and the fourth line did not express the minigene in any tissue examined. Thus, the FP1 mutation's deleterious effect is most pronounced in the gut, followed by the liver, and has the

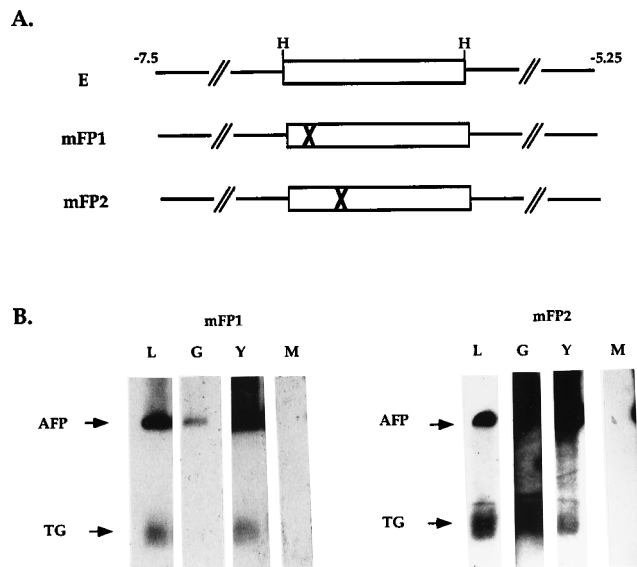


FIG. 7. Effects of mutations on activity of enhancer III in vivo. (A) The *Bam*HI-*Eco*RI fragment encompassing enhancer III (E) is depicted, along with the mutations (X) introduced into FP1 (mFP1) and FP2 (mFP2). The open box indicates the position of the 340-bp *Hinc*II (H) fragment containing the full activity of enhancer III. (B) RNA from transgenic e17 tissues (liver [L], gut [G], yolk sac [Y], and muscle [M]) were analyzed by agarose gel electrophoresis and hybridized to an AFP-specific probe which recognizes both the endogenous AFP (AFP) and the minigene RNAs (TG). The lines shown are mFP1-3 and mFP2-2.

smallest effect on the expression of the gene in the yolk sac. Consistent with an important role for FP1 in the gut and liver, high-level FP1 binding activity was detected in nuclear extracts from human Caco-2 cells, a gut-derived cell line, as well as in adult mouse liver and brain extracts (Fig. 8). Lower levels of FP1 were detected in the lung, and no activity was observed in the spleen. From the results of these experiments, it is clear that both FP1 and the HNF-3 site contribute to the developmental stage- and tissue-specific regulation of AFP in vivo.

DISCUSSION

The analysis of enhancer III was undertaken to examine its tissue specificity of action and to determine whether the high-level expression of the AFP gene in the visceral endoderm of

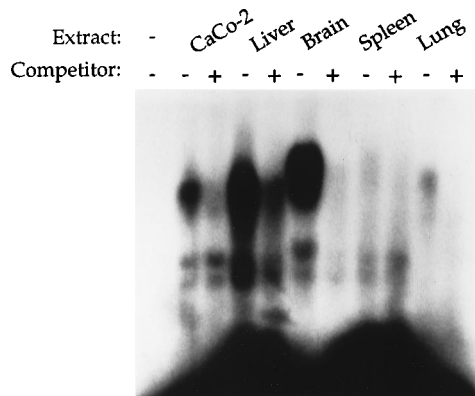


FIG. 8. Tissue distribution of FP1 binding activity. A double-stranded oligonucleotide containing FP1 was radiolabeled and incubated with the nuclear extracts indicated in the absence (-) or presence (+) of a 100-fold excess of unlabeled FP1 oligonucleotide. The complexes were displayed on an agarose gel.

the yolk sac could be explained by the fact that this enhancer, unlike the other two AFP enhancers, was selectively active only in that tissue and not in other endodermal derivatives. Our conclusion from these studies is that the enhancer has a broad endoderm specificity and that its action is not restricted to the visceral endoderm. Instead, the apparent tissue specificity is revealed only by mutations in the enhancer, which have less pronounced effects on enhancer activity in the yolk sac than in the liver or gut.

Like a number of other enhancers whose structures have been examined in detail, enhancer III is modular in nature. Three essential domains, A (1 to 140 bp), B (141 to 200 bp), and C (201 to 340 bp), were identified. The omission of any one resulted in either a complete loss of or reduction in enhancer activity. No region alone, even when duplicated, could reconstitute an active enhancer. This interdependency of the domains of enhancer III could be accomplished by several different mechanisms. First, the binding of one or more transcription factors might require the presence of other factors binding elsewhere in the enhancer. Alternatively, transcriptional activation might require a critical mass of independently binding proteins, which is not attained when portions of the enhancer are deleted. The fact that duplications of two of the domains, (III A+B)₂ and (III A+C)₂, result in partial and complete restoration of enhancer activity, respectively, suggests that the second model is more likely. In both instances, the duplicated regions contained region A.

From the outset, it was likely that region A played an important part in the activity of enhancer III. The original transgenic lines that displayed relatively weak AFP minigene expression were generated with a construct in which the first 50 bp of region A were missing (20). When this region, along with another 1 kb of DNA, was restored, the transgene was efficiently expressed in the fetal liver and gut as well as the yolk sac (Fig. 1). That this restoration was due to the 50 bp in region A and not additional elements in the flanking DNA is suggested by two lines of evidence. First, a small deletion of regions A to C (construct C in Fig. 1) completely eliminated enhancer activity, arguing that the extra 1 kb contained no additional enhancers capable of acting alone. More convincingly, a transgene in which a mutation was introduced into the 50 bp (mFP1; Fig. 7) exhibited a reduction in enhancer activity in the liver and gut similar to that observed with ZE.5/III (Table 1).

Despite the fact that either a deletion of FP1 or a site-specific mutation in its protein binding site was deleterious for enhancer activity *in vivo*, these mutations showed either no effect or a slight enhancing effect when tested by transfection in liver-derived tissue culture cells. In addition, when the binding site was tested alone or in tandem arrays, it displayed no independent enhancer activity, similar to other regulatory proteins such as LEF-1, which are thought to require a specific context of protein binding sites (15). The simplest explanation for the lack of effect of the FP1 mutation in transfections is the absence of the appropriate binding protein in HepG2 cells. This is the case for the liver-enriched factors of the C/EBP protein family, whose absence in HepG2 cells explains the failure to achieve high-level expression of the albumin gene in these cells (14). However, FP1 was identified by virtue of the fact that a liver-enriched protein found in HepG2 extracts bound to it *in vitro*. Either this protein is distinct from the one mediating the effects of FP1 in mice or it requires additional factors missing from HepG2 cells. The alternative, that FP1 is required for the assembly or maintenance of an open chromatin structure that would be detected only with integrated constructs, is considered below.

As we had observed for ZE.5/III transgenic lines, the mFP1

mutation had different effects on minigene expression in the three AFP-expressing tissues. The three lines which expressed the minigene did so in the yolk sac, suggesting that this tissue does not absolutely require the presence of FP1. In contrast, none of the lines expressed the gene in the fetal gut, suggesting that in that tissue, the site is essential. Whether this tissue difference reflects a different combination of transcription factors in the yolk sac than in the gut or merely a difference in the concentration of the same set of factors remains to be determined.

The core of FP1 is completely conserved in the rat homolog of enhancer III. In addition, a sequence very similar to FP1 (12-of-16-bp match) is present in enhancer I of both the mouse and rat genes (6, 17). However, no match was found in enhancer II, which is inactive in the gut. It is possible that FP1 plays a role in this tissue distinction between enhancers I and III, which display gut specificity, and enhancer II, which does not. A search through the regulatory regions of a number of other endoderm-specific genes revealed a similar sequence (match of 11 of 16 bp) at -541 bp in the rat albumin gene promoter, in a region which has not tested for functional activity.

Region A contained two additional sequences which bound liver-enriched proteins. One of these, FP2, was shown by mutation to have a central role in the overall activity of the enhancer in tissue culture cells. On the basis of the protected sequence, we reasoned that one or more members of the HNF-3 family of proteins was likely responsible for FP2 binding. This inference was confirmed by gel mobility shift assays, in which FP2 bound recombinant HNF-3 β and generated a gel shift pattern very similar to that obtained with HepG2 extracts. When the HNF-3 mutant was tested in tissue culture, it reduced enhancer activity 10-fold relative to wild-type levels, essentially abolishing enhancer function. *In vivo*, however, the effects were more complex, in that tissue-specific enhancer activity was maintained, although each animal displayed a unique pattern of expression.

The variability in the expression patterns of both the mFP1 and mFP2 lines is characteristic of a classical position effect, whereby the activity of a transgene is dependent on its site of integration. Such position effects have been generally considered to reflect a competition between transcription factors required to assemble an enhancer into an active configuration in chromatin and the nonspecific histone proteins (28, 38, 44). That competition will be affected by many factors, including the concentrations of transcription factors and their affinities for DNA in a given cell type, the modification state of the histones (33), and the effects of distant insulating sequences (27, 39, 43), which will be different for each transgene insertion. The fact that the yolk sac seems relatively impervious to mutations in FP1 may reflect the fact that this competition remains biased in favor of transcription factors in that tissue. In the liver and gut, the attenuation of one or more binding sites for factors is sufficient to bias the competition in the direction of the nonspecific nucleosome array at some integration sites while preserving expression at more permissive sites.

The position effects observed with the HNF-3 mutation support a recent suggestion that HNF-3 is involved in positioning nucleosomes on the mouse albumin enhancer (34). The authors showed that in liver nuclei, the enhancer exists in an array of three precisely positioned nucleosomes and that HNF-3 binding is required to assemble a comparable array *in vitro*. Furthermore, the recently reported structure of the DNA binding domain of HNF-3 γ (7) is similar to that of histone H5. An early role for HNF-3 (29) is consistent with the fact that first HNF-3 β and then HNF-3 α are expressed in

definitive endoderm following gastrulation in the embryo (2, 40), before the expression of other liver-enriched transcription factors.

These studies emphasize the value of testing the activity of site-directed mutations in vivo as well as by transient transfection (for example, see references 12, 24, and 37). The germ line transformation test is a stringent one, in that the regulatory elements are integrated into the host cell's chromosome and must be able to assemble into an active conformation from a repressed state during development as well as maintain high-level expression once the gene is activated (19, 23). We had argued previously that enhancers are absolutely required for this initial activation, given the requirement for at least one enhancer to activate the AFP minigene during embryonal carcinoma cell differentiation (49) and in transgenic mice (20). In contrast, enhancers are not absolutely required for either transient or stable expression in a fully differentiated cell (49).

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