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Developmental Regulation of α-Fetoprotein Genes in Transgenic Mice

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The mouse α-fetoprotein gene is activated in embryonic development in the visceral endoderm of the extraembryonic yolk sac and the fetal liver and gut. Transcription of the gene is subsequently repressed in the neonatal liver. To ask whether the DNA sequence elements required for tissue-specific activation are the same or different from those required for postnatal developmental regulation of the gene, modified copies of the α-fetoprotein gene were microinjected into fertilized mouse eggs. Those animals which developed to term and carried integrated copies of the modified gene were analyzed for expression. In approximately 50% of such animals, the introduced gene was active only in the three cell lineages which expressed the authentic α-fetoprotein gene. Furthermore, its expression was repressed in the neonatal liver. Thus, we conclude that the modified genes, which included either 7 or 14 kilobase pairs of 5′-flanking DNA, contained the DNA sequence information to direct both tissue-specific expression and developmental regulation. The observation that 50% of the mice which carried the modified gene did not express it in any tissue, combined with the fact that the level of expression was highly variable between expressing transgenic animals, suggested that the gene was susceptible to its site of integration in the mouse genome.

The orderly development of the mammalian embryo requires that appropriate sets of genes be activated in successive generations of differentiating cells. Throughout development cells must also maintain the ability to respond to signals which can modulate their patterns of gene expression. The selectivity of both processes is thought to reside, at least in part, within *cis*-acting DNA sequences that interact with tissue-specific *trans*-acting factors. With the ability to introduce cloned genes back into the germline of mice has come the opportunity to identify these DNA sequences and to begin to unravel the biochemical basis for tissue specificity and developmental regulation.

These issues are of particular interest in the case of genes that are expressed in more than one tissue, each of which regulates the gene in a different manner. A case in point is the murine α-fetoprotein (AFP) gene. This gene forms a small multi-gene family with the evolutionarily related serum albumin gene on chromosome 5 of the mouse (11, 18, 23). During embryonic development both genes are activated in concert in the visceral endoderm of the yolk sac, the fetal liver, and the fetal gastrointestinal tract (34, 42, 47). However, the levels of AFP and albumin mRNA are markedly different in these tissues, with the highest AFP levels accumulated in the visceral endoderm and the lowest levels accumulated in the gut. In addition, the AFP gene is differentially regulated in the liver relative to the albumin gene in that its rate of transcription undergoes a 10,000-fold decline shortly after birth (42). This decline is under genetic control by at least one *trans*-acting regulatory locus, termed *raf*, which determines the adult basal level of AFP mRNA (4, 27, 28).

To approach the molecular mechanisms underlying the regulation of the AFP gene in the visceral endoderm of the yolk sac, fetal liver, and fetal gut during development, it is important to determine whether the same DNA sequences are required for activation of the gene in each tissue and whether these sequences are the same as or different from those required for the postnatal developmental regulation in the liver. In a recent study, a transfected AFP gene was shown to be activated during retinoic acid-induced differentiation of F9 embryonal carcinoma cells into visceral endoderm (36). However, the response of the gene in the other tissues and during development cannot be ascertained in cell systems. Transgenic mice provide the ideal experimental system for addressing these questions of gene activation because they allow a detailed analysis of regulated gene expression in all tissues throughout development. For this reason, modified AFP minigenes were introduced into the germline of mice via the microinjection of fertilized eggs. Recent studies demonstrated that mice which carry microinjected genes integrated into nonhomologous chromosomal locations can proceed through development and transmit the exogenous DNA as Mendelian markers to progeny (5, 7, 10, 44). Several introduced genes in transgenic mice are not expressed at significant levels (16, 24); however, at least some genes are efficiently expressed (29, 30) and display the appropriate pattern of tissue-specific expression (6, 13, 25, 39, 40). We describe here the results of a study of transgenic mice carrying AFP minigenes. Our analysis shows that the exogenous AFP genes are expressed only in the appropriate fetal tissues and are, in addition, developmentally regulated in the liver after birth.

MATERIALS AND METHODS

Minigene constructs. The AFP minigenes YZE and ZE were constructed by joining two (Z and E) or three (Y, Z, and E) subcloned genomic *Eco*RI fragments from the albumin-AFP locus (Fig. 1A), producing a five-exon AFP minigene containing the first three and last two exons of AFP (36). The fragments were inserted into the *Eco*RI site of pBR322, their orientation was verified by restriction analysis, and the plasmid DNA banded twice in CsCl was used for microinjection. The five-exon ZE minigene when placed in the simian virus 40/pBR322 vector pSV2 and transfected into HeLa cells (35)

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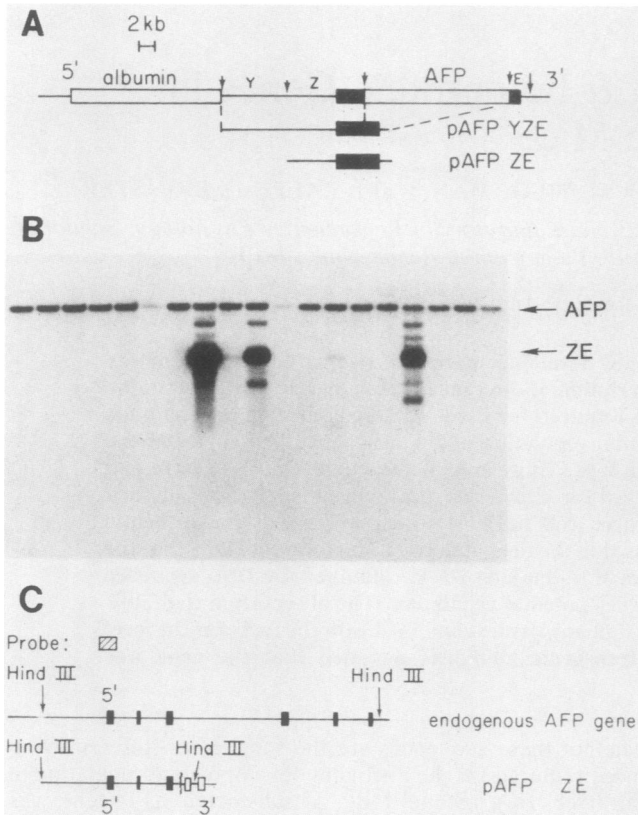

FIG. 1. Structure of mouse AFP minigenes and identification of transgenic mice by analysis of yolk sac DNA. (A) AFP minigenes YZE and ZE were prepared by using cloned Y, Z, and E *Eco*RI restriction fragments, indicated by the arrows, from the albumin-AFP locus (18, 23). The five-exon ZE minigene is generated by joining the Z fragment, containing 7 kb of 5'-flanking DNA and the first three exons of AFP, to the E fragment, containing the last two exons of AFP and 0.4 kb of 3'-flanking DNA. The five-exon YZE minigene contains an additional 7 kb of 5'-flanking DNA. These constructs have been cloned into the *Eco*RI site of pBR322. (B) Southern blot screen for mice carrying ZE minigenes. Yolk sac genomic DNA was isolated from 19 fetuses that developed from eggs microinjected with ZE minigenes, and 5 μg was digested with *Hin*dIII. The DNAs were hybridized after electrophoresis on a 1% agarose gel and transfer to nitrocellulose to an AFP genomic DNA probe spanning the first exon. (C) Diagram showing the basis for discrimination between the endogenous and exogenous DNAs, where the probe recognizes a 10-kb *Hin*dIII fragment in the endogenous gene and a 5-kb fragment in YZE or ZE.

produced a 600-nucleotide polyadenylated [poly(A)$^+$] RNA which was used as a standard for minigene transcripts.

Microinjection of fertilized mouse eggs. Transgenic mice were produced by using the YZE and ZE minigene plasmids linearized at the unique *Sal*I site in pBR322. The male pronucleus of F2 hybrid eggs (obtained by mating C57B1/6 × SJL hybrid adults) was microinjected with 2 pl containing approximately 260 copies of YZE or 360 copies of ZE. The injected eggs were reimplanted into pseudopregnant mice as previously described (5, 6) and allowed to develop for 18 to 19 days, at which time the fetuses and their yolk sacs were removed by caesarean section and then frozen. For the developmental studies, microinjected fertilized eggs were permitted to develop to term. The pups were delivered by caesarean section and given to foster mothers, and the yolk sacs were frozen for analysis.

Isolation of nucleic acids. Tissues were dissected from frozen embryos rapidly thawed in 0.9% NaCl. DNA and RNA were isolated from the fetal tissues by homogenizing the tissue in equal 1-ml volumes of water-saturated phenol containing 10% *m*-cresol, 0.1% 8-hydroxyquinolate, 50 mM Tris hydrochloride (pH 9.0), 1 mM EDTA, 1% sodium dodecyl sulfate, and 0.1% heparin (19). The aqueous layer was recovered by centrifugation for 10 min at 4°C in microfuge tubes, and the phenol phase was extracted. The aqueous layers were combined, adjusted to 0.15 M NaCl, extracted three times with a 0.5 volume of phenol, and then adjusted to 0.5 M NaCl. Nucleic acids were precipitated with 2 volumes of ethanol, and the DNA was removed by spooling and dissolved in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA. Total RNA collected by centrifugation was redissolved in 0.5 ml of 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–0.1% sodium dodecyl sulfate, and a portion was removed to determine the amount of poly(A)$^+$ RNA by a [^{3}H]polyuridylic acid-binding assay (33). The remaining RNA was enriched for poly(A)$^+$ by adding 10 μg of tRNA and 0.5 μg of polyadenylic acid as carriers, followed by chromatography on oligodeoxythymidylic acid-cellulose (2).

DNA was obtained from sections of mouse tails as described by Palmiter et al. (29). Approximately 40 mg of each tail was homogenized in 4 ml of 1% sodium dodecyl sulfate–5 mM EDTA–10 mM Tris hydrochloride (pH 8.0) containing 50 μg of proteinase K per ml and incubated at 37°C for 16 h. DNA was isolated by phenol extraction and ethanol precipitation, washed in 70% ethanol, and suspended in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA. All restriction enzyme digests with tail or tissue DNA were performed in the presence of 4 mM spermidine according to the instructions of the supplier.

Hybridization probes. A hybridization probe specific for AFP was prepared by subcloning into pUC9 a 440-base-pair *Hin*cII fragment containing the entire first exon of AFP and a portion of the first intron. Purified insert was isolated by digestion with *Hin*cII, electrophoresis in a 5% polyacrylamide gel in 1 × TBE (90 mMTris-borate [pH 8.3], 4 mM EDTA), and electroelution. Fragments were labeled by nick translation. For RNA analysis, an SP6 single-stranded RNA probe (12, 26, 48) from the first coding block of AFP was prepared by subcloning the 440-base-pair *Hin*cII fragment into the pSP65 vector (Promega Biotec). A transcription reaction containing 1 μg of pAFP-SP65 vector linearized with *Hin*dIII, 30 U of RNasin, 10 U of SP6 RNA polymerase, and 100 μCi of [α-^{32}P]GTP (>400 Ci/mmol) in 20 μl of a solution containing 40 mM Tris hydrochloride (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 20 mM NaCl, 10 mM dithiothreitol, and 500 μM each of CTP, UTP, and ATP was incubated at 37°C for 1 h. The DNA template was removed by digestion with DNase I at 20 μg/ml for 10 min at 37°C, stopped by the addition of EDTA and tRNA, and then extracted with phenol. The unincorporated label was removed by chromatography on Bio-Gel A1.5 agarose beads (Bio-Rad Laboratories) in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–0.1% sodium dodecyl sulfate, and approximately 0.75×10^8 to 1.5×10^8 cpm per reaction was obtained.

Nucleic acid analysis. DNA from tails and tissues was digested with restriction enzymes and transferred to nitrocellulose, after electrophoresis in agarose gels by the method of Southern (37). Poly(A)$^+$ mRNAs were electrophoresed in a 6% formaldehyde–1.7% agarose gel in 1 × MOPS buffer (pH 7.0; 20 mM morpholine propanesulfonic acid, 5 mM

sodium acetate, 1 mM EDTA) after denaturation at 55°C for 20 min in 70% formamide–6% formaldehyde–1× MOPS buffer and then transferred to nitrocellulose directly in 3 M NaCl–0.3 M sodium citrate (41). Filters were hybridized in 50% formamide containing dextran sulfate as previously described (45), except that hybridizations with the single-stranded RNA probe were performed at 57°C.

Quantitation of hybridization results was obtained from autoradiographs by densitometric analysis of different exposures. DNA copy numbers of the minigenes were based on the endogenous AFP signal assuming 2 copies per cell. The level of minigene mRNA represents the percentage of RNA relative to the endogenous AFP mRNA present in each sample. The number of minigene mRNA molecules per cell in the yolk sac was calculated by assuming that the yolk sac has 10 pg of RNA per cell and that the endogenous AFP mRNA comprises 20% of the poly(A)$^+$ RNA, equivalent to 42,000 molecules per cell (22). The numbers in Table 1 are a minimal estimate of molecules per cells because AFP is only made in the visceral endoderm layer of the yolk sac, and we harvested the entire yolk sac, which also contains mesoderm cells.

Primer extension. The primer (300 ng) was 5′ end labeled in a volume of 45 μl containing 300 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham Corp.), 70 mM Tris hydrochloride (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 6 U of polynucleotide kinase at 37°C for 60 min. The primer was extracted with phenol and ether, and then precipitated with carrier tRNA and 2 volumes of ethanol. The RNA (30 ng) and 10 ng of primer were resuspended in 10 μl of water, heat shocked at 95°C for 1 min, adjusted to 16 μl containing 140 mM KCl, 100 mM Tris hydrochloride (pH 8.3), 10 mM $MgCl_2$, 10 mM dithiothreitol, and 30 U of RNasin, and incubated at 37°C for 90 min. The primer was extended for 60 min at 37°C after adding 1 μl of 20 mM dXTPs and 1 μl of AMV reverse transcriptase (15 U; Life Sciences, Inc.). Reactions were phenol and ether extracted, precipitated with ethanol, washed once with 95% ethanol, and suspended in 12 μl of 80% formamide–1× TBE (90 mM Tris-borate [pH 8.3], 4 mM EDTA)–0.1% bromophenol blue–0.1% xylene cyanol. Samples were heated at 90°C for 3 min, cooled on ice, and electrophoresed on a 7 M urea–5% polyacrylamide gel in 1× TBE. The gel was soaked in 10% methanol-acetic acid for 30 min, vacuum dried for 2 h, and autoradiographed.

RESULTS

Microinjection of modified copies of the AFP gene. Two cloned five-exon AFP minigenes (36) that differ only in the amount of 5′-flanking DNA were chosen for microinjection into mouse eggs. The YZE minigene has 14 kilobases (kb) of 5′-flanking DNA which includes the entire intergenic region between the albumin and AFP genes, whereas the ZE minigene has 7 kb of 5′-flanking sequence (Fig. 1A). When introduced into cells, this five-exon minigene should generate a correctly initiated and fully processed poly(A)$^+$ transcript approximately 600 nucleotides (nt) long, with the potential to code for a protein 100 amino acids in length. Thus its mRNA can be readily distinguished from the endogenous 2.2-kb AFP mRNA on the basis of size, which allows us to directly compare the level of expression of the exogenous and endogenous AFP genes.

These plasmids were linearized at the unique *Sal*I site in the pBR322 vector and microinjected into the male pronucleus of fertilized eggs obtained from a cross between C57B1/6 × SJL mice as described above. The surviving eggs were reimplanted into the reproductive tract of pseudopregnant random bred Swiss females (5). In our initial experiments, gestation was allowed to continue for 18 to 19 days, at which time all progeny and their corresponding yolk sacs were removed by caesarean section and frozen. Genomic DNA was isolated from the yolk sacs and used to identify those animals which carried the microinjected DNA. The DNAs were digested with *Hin*dIII, separated by gel electrophoresis, and prepared for hybridization by the method of Southern (37). A labeled probe containing the first exon of the AFP gene detects a 10-kb *Hin*dIII fragment in the endogenous AFP gene and a 5-kb fragment in YZE or ZE (Fig. 1C). Figure 1B is an illustration of a screen of 19 yolk sac DNA samples, of which 5 contained an intact 5-kb ZE fragment. Three of the five contained multiple copies of the minigene, ranging from 6 to ~30 copies per cell, whereas the other two mice had less than 1 copy per cell and were subsequently shown to be mosaic for the exogenous DNA.

TABLE 1. Analysis of transgenic mice carrying AFP minigenes[a]

Minigene construct	Mouse	Minigene copies/cell	Level of minigene mRNA in yolk sac (% of endogenous AFP)	Minigene mRNA in yolk sac (molecules/cell)
YZE	5–2	4	8	3,100
YZE	39–6	12	1	420
YZE	42–1	0.5	0.2	80
ZE	50–2	10	25	10,500
ZE	28–4	2	10	4,200
ZE	47–3	12	8	3,400
ZE	35–7	8	4	1.700
Ze	47–4	40	3.4	1,400
ZE	47–2	50	2.2	920
ZE	32–3	6	2	840
ZE	55–6	1	1.7	710
ZE	54–4	70	1.5	630
ZE	54–3	70	0.9	380
ZE	55–5	50	0.6	250
ZE	47–1	15	0.1	40

[a] DNA copy numbers and relative levels of minigene mRNA were derived from autoradiographs by densitometric analysis of different exposures, with the endogenous AFP gene and mRNA levels as internal standards. The endogenous AFP mRNA comprises 20% of the yolk sac poly (A)$^+$ RNA, equal to 42,000 molecules per cell. The YZE minigene has 14 kb of 5′-flanking DNA, whereas the ZE minigene has 7 kb of 5′-flanking DNA. The ratio of mice positive for the DNA of each minigene was: YZE, 8/51; ZE, 26/109. The number of DNA positive mice does not include 12 mosaic animals also observed. The ratio of mice expressing the RNA of each minigene was: YZE, 3/8 (37%); ZE, 12/26 (46%).

Approximately 25% of the mice at term carried exogenous YZE or ZE DNA, with the copy number ranging from 1 to >70 copies per diploid genome (Table 1). The copy number was subsequently shown to be uniform in all somatic tissues of nonmosaic animals, with the multiple intact copies arranged as head-to-tail concatomers. The DNAs were typically integrated into single chromosomal locations and segregated as Mendelian markers, as has been described for other genes (5, 24, 30).

Tissue-specific expression of the AFP minigenes. The AFP gene is normally expressed in the visceral endoderm of the yolk sac, where its mRNA comprises approximately 20% of the total poly(A)$^+$ RNA (1, 22). To determine whether the AFP minigenes were being expressed in those animals that carried it, total poly(A)$^+$ RNA was isolated from the yolk sacs, size fractionated by electrophoresis in denaturing gels, transferred to nitrocellulose, and hybridized to a probe spanning the first coding block of the AFP gene. Significant

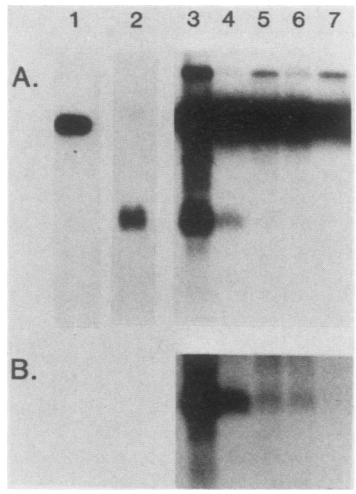

FIG. 2. Expression of YZE and ZE mRNAs in yolk sac RNA. Total poly(A)$^+$RNA (50 ng) from the yolk sacs of five selected transgenic animals was fractionated by electrophoresis through a denaturing formaldehyde agarose gel (1.7%) and transferred to a nitrocellulose filter. The filter was hybridized with a single-stranded RNA probe from the first exon of AFP (see the text) and autoradiographed for (A) 3 h or (B) 24 h (bottom half). Lane 1 contains 10 ng of mouse fetal liver poly(A)$^+$ mRNA as a standard for the migration of authentic 2.2-kb AFP mRNA, and lane 2 has 10 ng of 0.6-kb ZE mRNA produced by transient expression in HeLa cells. Lanes 3 to 7 represent RNA from transgenic animals 50-2, 47-3, 47-4, 39-6, and 54-3, respectively.

levels of a 600-nt RNA which comigrated with authentic minigene RNA were observed in 15 of 34 yolk sacs examined (Fig. 2, Table 1). This transcript also hybridized to probes derived from coding blocks 3 and 15 but not to cDNA probes derived from coding blocks 4 to 13, which are not represented in the minigene (data not shown).

The data (Fig. 2) serve to illustrate the variability in the levels of minigene RNA in the yolk sacs for both the YZE and ZE constructs. These range from 0.1 to 25% of the endogenous AFP mRNA, representing 40 to 10,500 copies of RNA per cell (Table 1). This variability does not correlate with the copy number of the DNA. That is, mouse 28-4 contains only 2 copies of ZE and accumulates ZE mRNA to 10% of the endogenous AFP mRNA level, whereas mouse 54-3 has >70 copies of ZE and accumulates ZE mRNA to 0.1% of the endogenous AFP mRNA level.

To determine whether the YZE and ZE minigenes were being expressed in other tissues, those 19-day-old fetuses producing minigene mRNA in the yolk sac were dissected into liver, gut, kidney, heart, and brain. Genomic DNA was isolated from the fetal tissues to ensure that each contained the same number of copies of exogenous genes originally observed for the corresponding yolk sac (data not shown). Total poly(A)$^+$ RNAs from the fetal tissues were fractionated by electrophoresis and hybridized to an AFP probe (Fig. 3). In addition to the yolk sac, authentic AFP mRNA is detected in fetal liver and to a lesser extent fetal gut but is not detectable in kidney, heart, or brain. The identical pattern of tissue specificity holds for the YZE mouse (39-6) and two ZE mice (28-4 and 35-7), since minigene transcripts were readily detected only in the yolk sac, fetal liver, and fetal gut RNAs. We used a mouse actin probe to confirm that all lanes had similar amounts of intact mRNA.

As observed in the yolk sacs, the levels of exogenous transcripts were generally lower than those of the AFP mRNA. In most cases, the yolk sac, liver, and gut in a single animal had levels of minigene mRNA that represent similar percentages of the endogenous AFP mRNA, as shown for mice 28-4 and 35-7 (Fig. 3). However, occasionally a discordancy was observed, where the levels of minigene mRNA in fetal gut more closely approximated those of the endogenous AFP mRNA. For example, in mouse 39-6 the relative levels of YZE mRNA were 250% of the endogenous level in fetal gut but only 1% of that in the yolk sac and 4% of that in the liver.

The results shown in Fig. 3 have been reproduced with 10

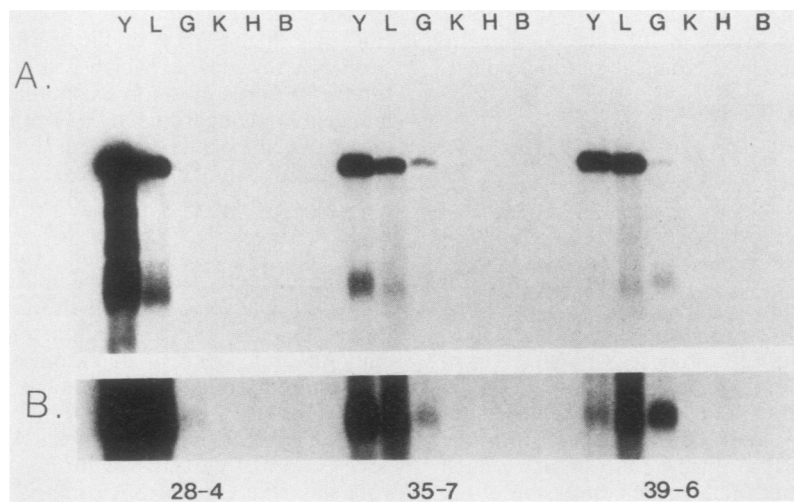

FIG. 3. Analysis of minigene expression in fetal tissues of three transgenic mice. Fetuses at days 18 to 19 of gestation were thawed in 0.9% NaCl and dissected into five tissues. Total poly(A)$^+$ mRNA was isolated from each tissue, and 15 to 30 ng of RNA from the yolk sac (Y), liver (L), gut (G), kidney (K), heart (H), and brain (B) was fractionated on 1.7% denaturing agarose gels by electrophoresis, transferred to nitrocellulose, and hybridized to an AFP-specific single-stranded RNA probe as detailed in the text. (A) 3-h exposure and (B) 36-h exposure of bottom half of the same filter. The upper band represents the authentic 2.2-kb AFP mRNA, and the lower band represents the 600-nt minigene mRNA.

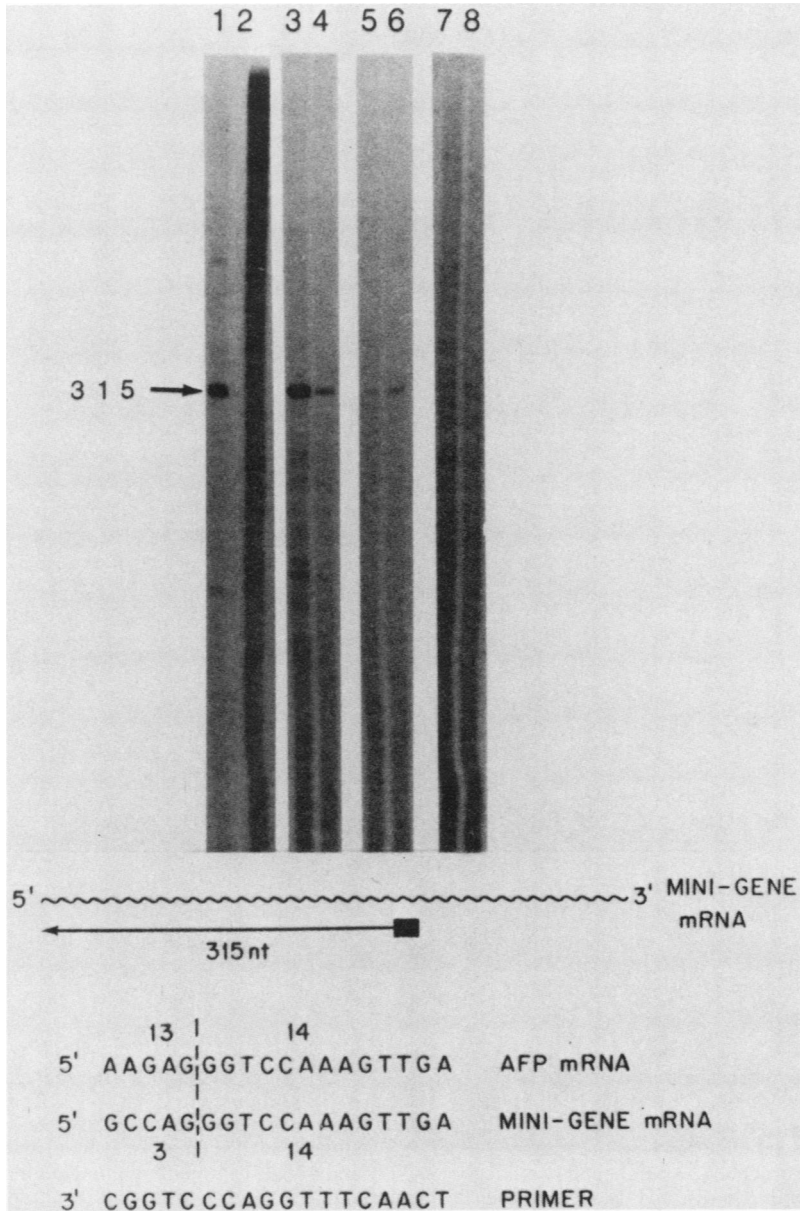

FIG. 4. Primer extension assay of ZE mRNA. The diagram at the bottom illustrates a synthetic oligonucleotide primer 18 bases long which is complementary to sequences at the exon 3-14 junction in minigene mRNA, located 315 nt downstream of the authentic AFP start site. The primer was associated with 30 ng of poly(A)$^+$ RNA and extended with reverse transcriptase. The RNAs used were derived from the yolk sac (lanes 3 and 4, mice 47-3 and 28-4), fetal liver (lanes 5 and 6, mice 47-3 and 28-4), and gut (lanes 7 and 8, mice 32-3 and 35-7). Lane 1 represents extension from 30 ng of minigene RNA obtained from transient expression of pZE in HeLa cells, and lane 2 represents extension from 20 μg of endogenous AFP mRNA from the yolk sac.

independent transgenic lineages. In addition, we looked at minigene expression in the tissues of the transgenic mice that did not express the minigene in our original yolk sac screen. None of these expressed YZE or ZE in any tissue. In summary, approximately 44% (15/34) of the transgenic mice carrying the AFP minigenes expressed significant levels of mRNA, always in a tissue-specific manner. The observation that the YZE and ZE constructs behave identically argues that the intergenic region between −7 and −14 kb of the AFP gene is not required for its tissue-specific expression.

Characterization of minigene RNA. The initial characterization of minigene mRNA was based on its predicted size and hybridization to coding block-specific probes. To directly demonstrate that the transcripts were initiated from the AFP cap site, a primer extension assay that was specific for the minigene mRNA was developed. A synthetic oligonucleotide primer 18 bases long complementary to the last 5 bases of exon 3 and the first 13 bases of exon 14 was prepared (Fig. 4). Such a primer will hybridize contiguously to minigene mRNA but not to endogenous AFP mRNA (Fig. 4, diagram). Reverse transcriptase should extend this primer only on minigene mRNA to generate a transcript 315 nt long if the proper initiation site is utilized. The appropriate 315-nt fragment is obtained with control RNA obtained from tran-

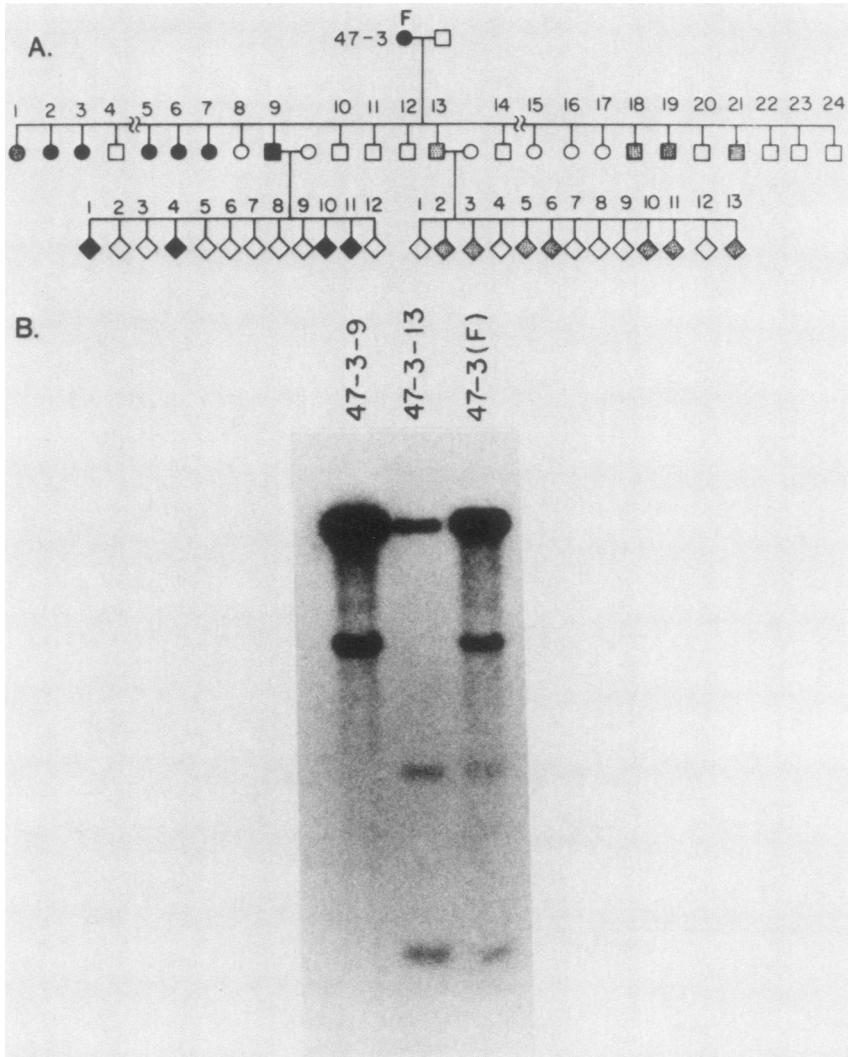

FIG. 5. Inheritance of ZE minigenes in the progeny of founder mouse 47-3. (A) Pedigree illustrating the segregation and inheritance of two different integration sites in the first two generations of 47-3 progeny. Mouse 47-3 was outbred to C57B1/6 × SJL males, and positive offspring were detected by tissue and tail blot analysis. Squares refer to males, circles refer to females, and diamonds refer to progeny sacrificed before birth. The solid symbols represent mice carrying 10 copies of ZE per cell, and the shaded symbols refer to mice with 2 copies of ZE per cell. (B) Blot of tail DNA from the original 47-3 parent and two offspring (47-3-9 and 47-3-13) with different integration patterns. A 20-μg amount of each sample was digested with *Hin*dIII, and the DNA was electrophoresed in a 1.3% agarose gel and then transferred to nitrocellulose. A 650-base-pair *Eco*RI/*Sal*I fragment of pBR322 representing one end of the linearized microinjected DNA labeled by nick translation was used to detect unique flanking chromosomal sequences in genomic DNA. The upper band in all three lanes represents a 5-kb internal *Hin*dIII fragment in the minigene constructs, and the lower bands represent junction fragments present in each animal.

sient expression of the minigene in HeLa cells (lane 1) but not with endogenous AFP mRNA from the yolk sac (lane 2) (Fig. 4). We analyzed the poly(A)$^+$ RNA from tissues of four transgenic mice (47-3, 28-4, 35-7, and 32-3) expressing the ZE minigene and detected a 315-nt fragment representing proper initiation in yolk sac, fetal liver, and fetal gut RNAs (Fig. 4, lanes 3 to 8). Similar results were obtained with RNAs from other transgenic mice. Therefore, ZE transcripts have the same 5′ start site as AFP mRNA in all tissues where the minigene is expressed. The relative intensities of the minigene signal in the different tissues agreed with those found by hybridization analysis (Fig. 3).

Developmental regulation of the AFP minigene in liver. The tissue-specific activation of the ZE minigene in transgenic mice could now be exploited to determine whether this construct contained sequences sufficient for its appropriate developmental regulation in the neonatal liver. Fertilized eggs microinjected with ZE constructs were allowed to develop to term, delivered by caesarean section, and given to foster mothers. Hybridization analyses of the yolk sac DNA and poly(A$^+$) RNA were used to identify those progeny which carried and expressed the minigene and therefore were of interest. Four such founder mice were mated, and male progeny carrying the ZE minigene were used to generate progeny for the developmental studies.

A pedigree for one of these animals (mouse 47-3) is shown in Fig. 5A. Offspring that inherited the injected gene from the original 47-3 female displayed two different patterns of integration of ZE DNA (47-3-9 and 47-3-13). Mouse 47-3-9 contained 10 copies per cell, whereas mouse 47-3-13 contained 2 copies per cell. Restriction enzyme digestion and hybridization analysis of the parent and two progeny DNA

showed that this was the result of the exogenous gene integrating at two independently segregating loci in the genome (Fig. 5B). A probe from the pBR322 vector hybridized to a common internal 5-kb *Hin*dIII fragment in all three animals, which represented the number of tandem copies of the minigene. However, mice 47-3-9 and 47-3-13 each contained a different subset of the lower-molecular-weight fragments present in the 47-3 parent, and these represented different junction fragments. To identify which of the integration sites was capable of expressing the minigene, yolk sac RNAs from the progeny of mice 47-3-9 and 47-3-13, which could now be considered as two independent lines, were analyzed by hybridization. All produced ZE transcripts and transmitted the minigenes as stable Mendelian markers to their offspring.

For the developmental studies, we first confirmed that the tissue-specific pattern of expression in a given line was stably inherited in all offspring. For this purpose F1 animals were obtained by caesarean section on day 18 of fetal development from five separate transgenic lines. In each of three lines (47-3-9, 47-3-13, and 55-6-18), every fetus that inherited the minigene expressed its mRNA in a qualitatively and quantitatively identical manner. Surprisingly in the two other lines (47-2 and 47-4), none of the offspring produced minigene transcripts. This apparently represents an inability of some integration sites to transmit expression through the germline, as has been observed for other genes (24, 29, 43).

The level of AFP mRNA is developmentally regulated in the mouse liver and declines rapidly after birth as a result of a decrease in its rate of transcription (42). Hepatic poly(A)$^+$ RNA was isolated on day 18 of gestation and at 3, 7, 14, and 28 days after birth from the three transgenic lines that were transmitting the expression of the minigene. The RNAs were analyzed by hybridization with the first exon AFP probe, and results for lines 47-3-9 and 47-3-13 at days 3, 7, and 14 are shown in Fig. 6. The minigene and AFP mRNA levels decreased more than 100-fold between day 3 and 14 and were not detectable by day 28. The relative rate of decline of both transcripts, as measured by densitometry, was identical, suggesting that both transcription rates were decreasing at the same time and to a similar extent. From this data, we conclude that the ZE minigene contains the regions required not only for tissue specificity but also for the postnatal transcriptional repression in the liver. The filters in Fig. 6 were also washed and rehybridized with an albumin cDNA probe which detected a relatively constant level of albumin mRNA in all samples and served as a control for the amount of RNA per lane (data not shown).

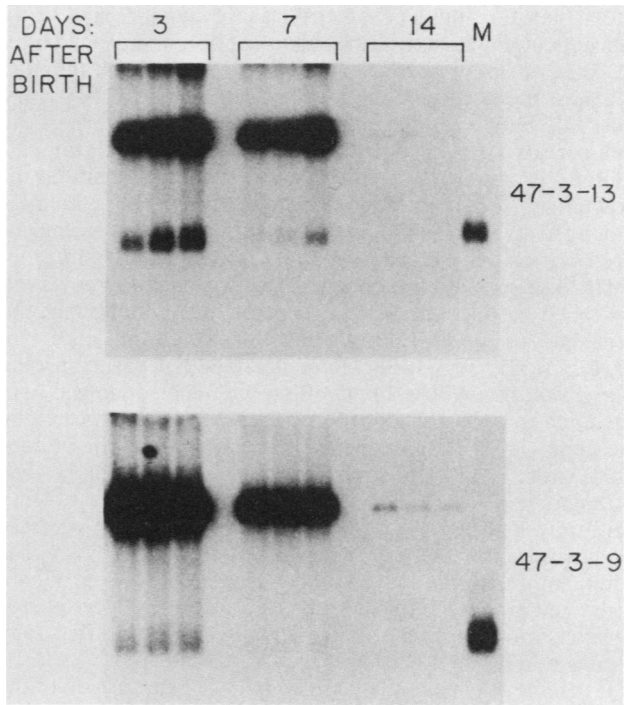

FIG. 6. Developmental modulation of the levels of minigene and AFP mRNA in the neonatal liver. Transgenic males 47-3-9 and 47-3-13 (Fig. 5) were mated to several females, and progeny were sacrificed at 3, 7, and 14 days after birth. Mice carrying minigenes were identified by DNA blot analysis, and poly(A)$^+$ mRNA was isolated from the corresponding livers. RNA (100 ng) from three animals at each of the time points was fractionated by electrophoresis in a denaturing agarose gel and transferred to a nitrocellulose filter. The filter was hybridized with a labeled single-stranded RNA probe homologous to the first AFP coding block, and relative levels of minigene and AFP mRNAs were determined by densitometric analysis of several autoradiographic exposures.

DISCUSSION

Transgenic mice provide an ideal approach to investigate the requirements for activation of genes in different tissues. At the same time, the response of activated genes to signals that modulate their activity during ontogeny can be studied. In the case of the AFP gene, this apparently complex interaction between tissue-specific factors and their response to humoral signals has been reproduced with the minigenes in mice. The results described above demonstrate that the AFP minigenes were efficiently expressed in approximately 44% of the mice which had at least one intact copy of the minigene integrated into their genome. The pattern of expression of the minigenes was qualitatively identical to the endogenous AFP gene in that minigene transcripts were detected in the yolk sac, fetal liver, and fetal gut but not in the kidney, heart, or brain. We have never generated an animal in which the minigene was expressed in an inappropriate tissue. Therefore, the minigene can be appropriately activated at a variety of chromosomal locations.

Expression of the ZE minigene was transmitted to progeny in three of five lines, and in all three cases expression was tissue specific. Thus we could ask whether the minigene mRNA was also developmentally regulated in the neonatal liver. The data (Fig. 6) clearly demonstrate that this was the case, in that the reductions in AFP and minigene RNAs followed a parallel course. This is the first instance in which a cloned gene introduced into the mouse genome has been shown not only to express in the proper tissues but also to respond to developmental signals that modify its pattern of expression. From this we conclude that the DNA signals necessary for developmental repression as well as for tissue-specific activation are contained in the DNA that has been introduced into mice. The precise location of these sequences and their degree of overlap, if any, must now be addressed. The observation that plasmid pZE, which contains 7 kb of 5'-flanking DNA, is able to confer tissue specificity upon the expression of the minigene as effectively as pYZE illustrates that it is possible to activate and modulate the AFP gene in the absence of the albumin gene and a substantial part of the intergenic region.

Two observations suggest that the chromosomal position

into which the minigene integrates is playing a role in its subsequent expression. First, transcription of pZE can only be detected in a subset (~44%) of the transgenic animals. Second, the steady-state levels of minigene RNA vary markedly between different animals, from 0.1 to 25% of the endogenous AFP level in the yolk sac (Table 1, Fig. 2). Neither property is a function of the copy number of exogenous DNA per haploid genome, since those animals which fail to express the minigene display the same range of DNA copies per cell as those that do express the minigene.

The influence of chromosomal position has been observed and used to account for the lack of and variation in the expression of several other genes introduced into mice (16, 20, 21, 24, 29, 30, 31, 38). Recently, low but tissue-specific expression of a mouse-human β-globin fusion gene was also observed in only a fraction of the transgenic mice carrying the gene (F. Costantini, personal communication). In contrast, nearly all transgenic mice carrying mouse immunoglobulin (6, 13, 39) or rat elastase (40) genes express uniformly high levels of the exogenous gene. One possible explanation for the efficient use of these genes is that dominant transcriptional enhancer sequences (3, 8, 9, 32, 46) contained in these constructs may be able to compensate for chromosomal position effects. Thus it is possible that they represent a distinct class of genes.

If the above is true, the variable levels of expression of the AFP minigenes in mice might be the consequence of the fact that not all genes contain these compensatory elements. One must then postulate that there is a different class of control element more dependent upon chromosomal position. This implies that a chromosomal environment spanning a large genomic domain may be required for appropriate expression, and these domains might be difficult to recreate. Therefore, the sensitivity of the gene to the site of integration may actually reflect normal control mechanisms distinct from the dominant enhancers. It may not be a coincidence that both the reintroduced AFP and β-globin genes (Costantini, personal communication) display inefficient or variable levels of expression in mice. Both are present within large genomic loci containing multiple genes whose transcription is being temporally regulated.

The alternative possibility to consider is that the AFP minigenes are susceptible to position effects because they lack critical transcriptional enhancer sequences, similar to those in the immunoglobulin or elastase genes that are present in the authentic gene. Constructs that contain additional internal and flanking sequences need to be tested to determine whether they can express levels of mRNA more closely resembling the endogenous AFP gene.

It is formally possible that missing an internal or flanking sequence may be required for the stability of the primary or mature transcript. Our results suggest that differential stability of the minigene transcript alone is not responsible for the relatively low levels of minigene mRNA in the yolk sac, liver, and gut, since this would have the identical effect in all transgenic lines, yet the levels of RNA vary dramatically. In addition, if the minigene RNA was substantially less stable than AFP mRNA, we would predict that minigene RNA levels would fall more rapidly when AFP transcription declines in the liver after birth. However, the relative ratio of the minigene mRNA to endogenous AFP mRNA does not change during repression in the postnatal period. Therefore, differences in the rates of transcription are more likely to be responsible for the differences between minigene and AFP mRNA levels.

Regardless of which of these possibilities is the case, it is important to note that only the level of expression, and not the tissue specificity, is affected by position. Therefore, in the minigene constructs the *cis*-acting DNA signals required for tissue specificity and developmental regulation have been dissociated from efficient transcription of the gene. This observation is consistent with results obtained in F9 teratocarcinoma cells. In these experiments, pYZE was completely repressed when introduced into the stem cells and was selectively activated upon formation of visceral endoderm but not parietal endoderm (36). However, the levels of minigene mRNA which accumulated in these cells were low relative to those of the endogenous AFP mRNA. The activation of the AFP gene in the visceral endoderm layer of aggregating F9 cells (17) is believed to occur through a multi-step process involving the initial activation of the gene during the formation of the visceral endoderm, followed by a secondary 200-fold induction as the endoderm associates with its basement membrane (14, 15). The pYZE minigene in F9 transformants was activated in the visceral endoderm at the appropriate time but failed to accumulate during the secondary induction, suggesting that the minigene lacked the necessary signals (36). By analogy, we suggest that similar multi-step processes are required for the maximal expression of AFP in mouse tissues, as reflected by the fact that the minigenes are not maximally expressed.

We observed several instances in which the relative levels of minigene RNA in each of the three tissues in a single animal are not identical. A particularly striking example of this is shown in Fig. 3, where the relative level of minigene RNA in the gut is significantly higher than the level in either the liver or the yolk sac. Likewise, several animals exhibited higher levels in the liver than in the yolk sac, in contrast to the endogenous mRNA. These unusual relationships might be explained by differences in the activity of chromosomal domains adjacent to the foreign gene in the different tissues (20). However, another intriguing possibility is that these fluctuations are indicative of different regulatory mechanisms used by these tissues to modulate the transcription of the gene. If that is so, then it may be possible, with the appropriate constructions in transgenic mice, to dissociate the expression of the minigene in each of the three cell lineages and to examine the DNA sequence requirements for both the activation and the developmental modulation of AFP in each tissue.

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ADDENDUM IN PROOF

We have recently introduced the ZE minigene free of plasmid vector DNA into fertilized eggs and observed high-level expression of the minigene MRNA in 19-day-old yolk sacs in 12 of 12 animals. Thus, the variability in the level of expression of the minigene reported here is primarily the consequence of interference by the plasmid DNA.

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