Diversity of Alpha-Fetoprotein Gene Expression in Mice Is Generated by a Combination of Separate Enhancer Elements

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The 5′ flanking region of the mouse alpha-fetoprotein (AFP) gene contains a tissue-specific promoter and three upstream regulatory elements that behave as classical enhancers. At least one of these enhancers is now shown to be required for the tissue-specific expression of the AFP gene when it is introduced into the mouse genome by microinjection of cloned DNA fragments into fertilized eggs. Each enhancer can direct expression in the appropriate tissues, the visceral endoderm of the yolk sac, the fetal liver, and the gastrointestinal tract, but each exerts different influence in these three tissues. These differences may explain the tissue-specific diversity in the levels of expression characteristic of the AFP gene. The postnatal repression of transcription of the AFP gene in both liver and gut, as well as the reinitiation of its transcription during liver regeneration, is mimicked by the introduced gene when it is linked to the enhancer domains together or singly. Thus, the DNA sequence elements responsible for directing the activation of AFP transcription, its repression, and reinduction are contained in a limited segment of DNA within or 5′ to the gene (or both) and are operative in the absence of the closely linked albumin gene.

THE ALPHA-FETOPROTEIN (AFP) GENE EXHIBITS A DIVERSE pattern of tissue specificity and temporal regulation during development in the mouse. It is activated, together with the evolutionarily related albumin gene (*1–5*), in three independent cell lineages: the visceral endoderm of the yolk sac, the fetal liver, and the fetal gastrointestinal tract (*6*). The AFP messenger RNA (mRNA) is maintained at very different steady-state levels in each tissue; it constitutes about 20 percent of the total mRNA in the visceral endoderm, about 5 percent in liver, and less than 0.1 percent in the gut (*6–8*). After the birth of the animal, AFP mRNA declines in the liver and gut, although genetic evidence suggests that the repression proceeds by different mechanisms in each tissue. That is, the adult basal level of AFP mRNA in liver is influenced by an unlinked gene termed *raf*, but this is not the case in the gastrointestinal tract (*8, 9*).

The biochemical basis for such diversity could include both multiple cis-acting DNA elements (*10, 11*) and multiple tissue-specific factors (*12, 13*) that differentially interact with them. Evidence in favor of multiple DNA elements has been obtained from experiments that depended on the transient introduction of modified AFP genes into a human hepatoma cell line (*14*). These studies disclosed four distinct regulatory elements beginning at the site of transcriptional initiation and proceeding 7.6 kb upstream of the gene. Three of these elements were separately shown to have properties of classical enhancers; they augmented transcription of the AFP gene in a manner independent of orientation and position, and were able to stimulate transcription from a heterologous promoter in hepatoma cells and to a lesser extent in HeLa cells. The activity of these enhancers in heterologous cells suggested that the enhancers were only partially responsible for the tissue specificity of the gene; indeed a segment of DNA, −85 to −52 nucleotides 5′ to the gene, was shown to be essential for transcription in hepatoma lines, but was not active in HeLa cells (*14, 15*).

When assayed by the transient expression system in hepatoma cells, the three enhancers had essentially equivalent and nonadditive activities, suggesting that they were functionally redundant. However, it is likely that such assays measure only a subset of the biological activity of a DNA segment. We tested the in vivo activity of these elements, together and separately, by introducing them linked to the AFP promoter region into the mouse germline. By this procedure the function of these genetic elements in establishing the tissue-specific pattern of gene expression in addition to the role (or roles) that they might play in the later developmental modulation of the AFP gene was assessed. In contrast to the transient expression assays, the three enhancer domains were not functionally equivalent in vivo, thus providing strong support for the hypothesis that diversity in gene expression is being accomplished at least in part by diversity at the level of cis-acting DNA regulatory elements.

High level expression of the AFP gene in transgenic mice. We have already reported the generation of transgenic mice that carry an AFP "minigene" consisting of the first three and the last two AFP exons, along with either 13.5 or 7.6 kb of 5′ flanking DNA and pBR322 vector sequences (*16*). Only 50 percent of the mice expressed the introduced gene, and the levels of mRNA expression varied widely. For example, in the yolk sac the range was from only 0.1 to 25 percent of endogenous AFP mRNA levels. Data from

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several laboratories (*17–19*) had suggested that such variability could arise from the presence of the prokaryotic vector DNA. To find out whether this was the case for the AFP minigene, we performed the following experiments. Gel-purified AFP minigene fragments containing 6.6 kb of 5′ flanking sequences (ZE) were introduced into mice by microinjection of fertilized eggs (Fig. 1). Many transgenic animals were generated as a means of resolving the inherent biological activity of each construct from the effects of chromosomal position and copy number. On day 18 of gestation, the foster mothers were killed, and all fetuses and corresponding yolk sacs were removed and frozen. Genomic DNA was isolated from individual yolk sacs (*16*) and Southern blot analysis (*20*) was used to identify the animals that carried the microinjected DNA. The tissue-specific expression in transgenic animals was analyzed by Northern blotting of poly(A)$^+$ RNA prepared from six different tissues (*16*). The minigene generates a small polyadenylated, 600-nucleotide-long mRNA which was readily distinguished from the 2.2-kb authentic AFP mRNA (*21*).

The AFP minigene mRNA was present in the yolk sac, at levels approximating that of the endogenous gene in all 12 ZE-bearing transgenic mice; these concentrations ranged between 20 and 125 percent of the endogenous AFP mRNA concentration (Fig. 1). No correlation between the expression and copy number of the integrated DNA was observed in this tissue. Thus, the low frequency of transgenic animals that expressed the AFP minigene in previous experiments was due to interference by plasmid DNA rather than a susceptibility to the site of integration. The degree to which negative interference by plasmid DNA occurs appears to be a gene-specific phenomenon in that the genes for rat elastase (*22*) and mouse immunoglobulin (*23–25*) do not show this effect to any significant extent whereas the mouse and human β-globin genes are very sensitive (*17, 18, 26*). The reasons for this sensitivity are not well understood, but we cannot attribute it solely to the absence of strong cellular enhancers, because the AFP minigene construct tested previously (*16*) contained such elements and was relatively sensitive to the presence of the vector DNA. In any case, elimination of the vector reduced the variation and increased the expression of the minigene so that we were able to map the DNA elements responsible for establishing the levels of expression in various tissues as well as the developmental postnatal regulation.

Expression of the ZE minigene was restricted to the expected tissues, the yolk sac, liver, and gut (Fig. 1B), consistent with our previous observations (*16*). However, the levels of minigene expression in the liver and especially the gut exceeded those of authentic AFP mRNA. Similar data were obtained for all the transgenic animals carrying the ZE minigene (Fig. 1C). Thus with respect to tissue specificity, the AFP minigene mimics the endogenous gene qualitatively, but not quantitatively.

Whether the AFP minigene could be activated in the absence of the three enhancer domains between −1 and −6.6 kb was determined as follows. A minigene containing only 1 kb of 5′ flanking DNA, termed ZE.5, was introduced into fertilized eggs, and the progeny were examined for the presence of exogenous DNA and its expression in term fetuses. No expression was observed in any of the animals investigated (Table 1). This result is consistent with preliminary results obtained with stably transfected F9 teratocarcinoma cells (*27*). In that instance, the enhancers were required for the activation of transcription of the minigene during differentiation. From these results, we conclude that the enhancer domains are necessary to initiate expression of the minigene during development.

Table 1. Expression of AFP minigenes with 14 kb and 1 kb of 5′ flanking DNA in transgenic mice. Various tissues from transgenic mice killed at day 19 of gestation were analyzed for DNA copy number and for AFP mRNA expression (legend to Fig. 1). Densitometry of autoradiograms was used to estimate the amount of AFP minigene mRNA present relative to the endogenous AFP mRNA in that tissue. No expression was observed in heart or brain of any animal. ZE.5 contains 1 kb of 5′ flanking DNA. It was cleanly excised from the vector sequences as a Bam HI–Eco RI fragment. Four additional mice containing less than one copy of the ZE.5 DNA fragment were examined and were also negative for expression in yolk sac. YZE contains the entire 14-kb intergenic region between the albumin and AFP genes fused to the AFP minigene. The Eco RI fragments Y, Z, and E (*30*) were reassembled in a pUC 19 derivative that had been engineered by synthetic oligonucleotide cloning to contain Sal I sites on each side of the polylinker-cloning region so that the entire 17.4-kb fragment, containing 33 and 12 nucleotides of polylinker DNA on the 5′ and 3′ ends, could be excised with Sal I.

Construct	Mouse	DNA copy number	Endogenous AFP mRNA in			
			Yolk sac (%)	Liver (%)	Gut (%)	Kidney
ZE.5	126-8	1	0	0	0	–
	129-4	1	0	0	0	–
	129-5	1	0	0	0	–
	130-4	1	0	0	0	–
	106-2	1	0			
	111-2	2	0			
	113-2	2	0			
	108-6	4	0			
YZE	225-3	2	17	24	250	–
	225-8	2	24	605	1320	
	228-12	2	22	256	475	+
	224-3	6	52	233	390	+
	224-5	6	2	8	0	–
	229-10	~40	74	320	>5000	++

Table 2. Expression of AFP minigenes with the three enhancer domains singly or in combination in transgenic mice. The DNA copy number and AFP minigene mRNA analyses were determined as in the legend to Fig. 1 for the constructs shown in Fig. 2. No expression of the AFP minigene was observed in heart or brain for any of the mice listed. N.A., not analyzed.

Construct	Mouse*	DNA copy number	Endogenous AFP mRNA in			
			Yolk sac (%)	Liver (%)	Gut (%)	Kidney
ZE.5/I	137-3	2	9	0	258	N.A.
	134-5	3	8	10	502	N.A.
	138-8	3	2	0	>5000	+
	138-7	5	4	80	>5000	++
	138-6	10	28	152	>5000	++
	134-3	10	50	308	>5000	++
	134-2	10–11	51	80	2800	++
	142-4	>50	59	81	>5000	++
ZE.5/II	184-2	8	6	4	0	+
	185-3	20	58	102	180	++
	189-2	25	0	0	0	–
	188-1	50	50	31	155	++
ZE.5/III	205-2	4	9	12	94	++
	194-1	7	24	2	0	+
	194-3	16	19	0	0	+
	193-1	40	52	0	0	–
	209-3	~100	21	4	149	++
	210-3	~100	27	4	15	++
	212-7	~100	1	0	0	++
	208-3	>100	20	2	34	++
ZE.5/II+III	154-2	2	3	0	10	–
	163-5	5	3	10	0	–
	152-5	~6	6	100	82	++
	151-2	80	150	37	73	++

*The numbers designate the mouse.

Tissue specificity of the AFP regulatory elements. To address the possibility that the multiple enhancer domains in the intergenic region contribute to the diversity of gene expression, each was attached separately or in combination to the inactive ZE.5 minigene. These constructs were introduced as isolated fragments into fertilized eggs, and representative examples of the tissue-specific pattern of RNA expression observed in full-term fetuses carrying them are shown in Fig. 2. The elements varied in strength among the different tissues but always directed expression to the appropriate ones. The attachment of element I (−1.0 to −4.1 kb) to ZE.5 restored activity of the minigene to a pattern of expression similar to that obtained when all three elements were present (compare Fig. 1B and Fig. 2, panel A). High level expression was observed in the gut in all transgenic animals. The levels in yolk sac and liver were generally comparable to those in ZE mice, although there was a reduction in the steady-state level of minigene mRNA in liver (compare Fig. 1 and Table 2), suggesting this enhancer was not sufficient for full activity in liver.

Animals carrying either element II (−3.8 to −5.4 kb; Fig. 2B) or element III (−5.4 to −6.6 kb; Fig. 2, panel C) did not contain the very high minigene mRNA levels in the gut which characterized the expression in the ZE and ZE.5/I mice (Table 2). For those animals carrying ZE.5/III, expression was very similar to those with ZE.5/II, except that the levels of minigene mRNA in liver were lower than for any other construct. The combination of elements II and III (Fig. 2 and Table 2) yielded a level of expression that was characteristic of element II alone, suggesting that the activities of these two elements were not additive.

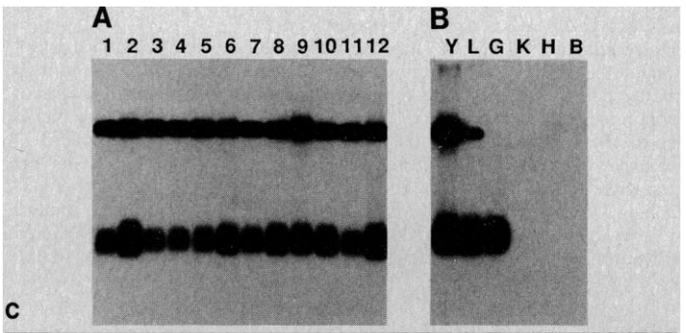
These results suggest that while each of the enhancer elements had equivalent biological activity in hepatoma transient expression assays (*14*), they were not functionally equivalent in vivo. Each element differed in its capacity to direct expression in the liver, with element I≥II>>III. In the gut, the elements varied dramatically, with I>>>II=III.

Furthermore, taken singly, these elements were sensitive to the site of integration whereas, when together, position effects were less striking. Position effects, that is, significant variation in the expression of the minigene between independently derived animals, were most pronounced with animals carrying either element II or III. The effects of position on expression were not the same for each tissue. For example, animal 138-8 (ZE.5/I, Table 2) expressed the minigene at very high levels only in the gut; animal 152-5 (ZE.5/II+III) showed expression of the minigene comparable to the endogenous mRNA in the liver and gut, but not in the yolk sac. These data suggest that the heterologous chromosomal location is exerting different influences in the three cell lineages.

In more than 50 percent of the mice, the expression of the minigene in the fetal kidney was low (Table 2). The endogenous AFP gene expression in that tissue is also very low, at least ten times lower than in the gut, but the amount of the minigene was at least an order of magnitude greater than that of the endogenous AFP gene in some cases. There was no obvious correlation between this expression and either the copy number of the fragment, or its level of expression in other tissues. Significant overexpression in kidney was occasionally observed with all constructs tested, unlike overexpression in the gut, which depended on the presence of element I.

Overexpression of the AFP minigene. The element I–dependent high level of minigene expression in the gut was unexpected. There was a general trend for overexpression in liver and gut to be dependent on copy number for both ZE and ZE.5/I, but it was not an invariant correlation because mouse 146-11, which carried only a single copy of the minigene, contained transcripts in gut at 20 times the endogenous level.

The molecular basis for overproduction of the minigene in the gut has not been defined, but the data favor the possibility that it is primarily transcriptional, because overproduction is dependent on the presence of a transcriptional control signal, element I. The ability of the minigene to accumulate transcripts in the gut to the same level as in the yolk sac certainly suggests that trans-acting factors are not limiting in either tissue. Rather, the data argue that AFP transcription in the gut is normally limited by the accessibility of the gene in that tissue to excess transcription factors. Accordingly, in the fertilized egg the minigenes have integrated into relatively "accessible" sites that remain so in all subsequent cell lineages. Inaccessibility of the endogenous gene could be achieved by various mechanisms,



Mouse	DNA copy number	Endogenous AFP mRNA in			
		Yolk sac (%)	Liver (%)	Gut (%)	Kidney
146-11	1	64	128	2075	+
145-4	2–3	61	66	600	+
146-7	3–5	30	93	475	+
143-2	6–8	27	86	4930	+
147-3	12	49	319	1660	−
147-5	~50	20	275	>5000	+
143-3	~50	60	200	>5000	+
149-2	~50	65	950	>5000	++
149-7	~70	125	670	>5000	+
147-4	~80	87	775	>5000	++

Fig. 1. Tissue distribution and levels of expression of the AFP minigene ZE. F_2 hybrid embryos from C57BL/6 × SJL/J parents were injected with a purified DNA fragment carrying the AFP minigene (*16, 20*) and 6.6 kb of 5′ flanking DNA (Fig. 2) as described by Brinster *et al.* (*34*). DNA was prepared by electroelution of a Cla I fragment from an agarose gel and subsequent glass-powder binding (*35*). The DNA concentration was determined by a sensitive fluorescence method (*36*) and diluted to 2 ng/μl for microinjection. Injected eggs were transferred to pseudopregnant mice. At day 18 to 19 of gestation, fetuses were collected and frozen. RNA and DNA were prepared from yolk sac and transgenic animals were identified by Southern blot analysis of yolk sac DNA (*16, 20*). Total poly(A)$^+$ RNA was isolated from the yolk sacs of 12 transgenic fetuses (**A**). RNA representing 2 percent of the total yolk sac poly(A)$^+$ RNA and 15 percent of the poly(A)$^+$ RNA for somatic tissues was run on 1.5 percent formaldehyde agarose gels and blotted onto nitrocellulose (*16*). Endogenous AFP mRNA (upper bands) and minigene mRNA (lower bands) were detected with an AFP exon 1–specific probe on Northern blots (*16, 37*). The tissue-specific distribution of the minigene in a typical ZE line (143-3) is shown in **B**, for yolk sac (Y), liver (L), gut (G), kidney (K), heart (H), and brain (B). Estimates of minigene mRNA levels relative to endogenous AFP levels (**C**) were based on densitometric scanning of autoradiograms from Northern blots of poly(A)$^+$ RNA isolated from the tissues of ten different mouse lines. The levels of minigene mRNA in yolk sac, liver, and gut are expressed as a percentage of the endogenous AFP mRNA in that tissue. The levels in kidney are expressed as minigene mRNA not detected (−), detected on the same order of magnitude as the endogenous kidney mRNA (+), or at a level more than five times greater than endogenous AFP expression in kidney (++). No minigene expression was detected in heart or brain. DNA copy number per cell was estimated from autoradiographs of Southern blots on the assumption that the endogenous AFP signal represents two copies per cell. Mice that did not have the same DNA copy number in yolk sac, liver, and gut were judged to be mosaic and are not included in the table.

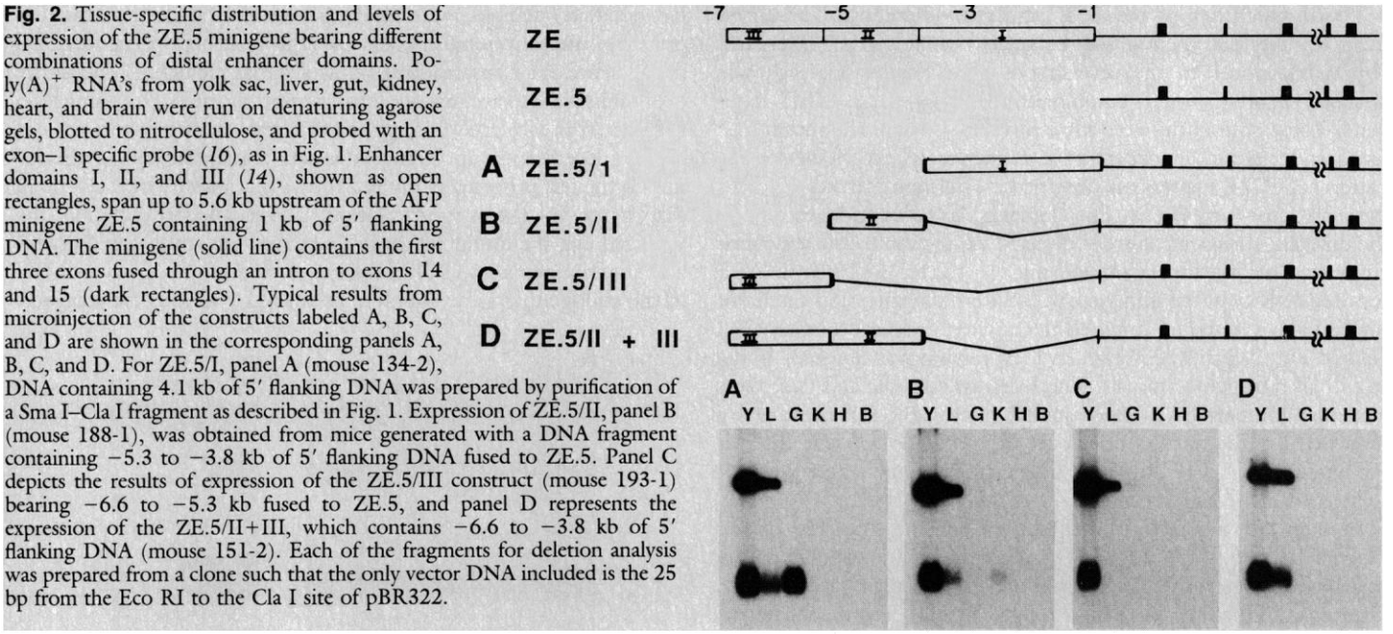


Fig. 2. Tissue-specific distribution and levels of expression of the ZE.5 minigene bearing different combinations of distal enhancer domains. Poly(A)$^+$ RNA's from yolk sac, liver, gut, kidney, heart, and brain were run on denaturing agarose gels, blotted to nitrocellulose, and probed with an exon–1 specific probe (*16*), as in Fig. 1. Enhancer domains I, II, and III (*14*), shown as open rectangles, span up to 5.6 kb upstream of the AFP minigene ZE.5 containing 1 kb of 5′ flanking DNA. The minigene (solid line) contains the first three exons fused through an intron to exons 14 and 15 (dark rectangles). Typical results from microinjection of the constructs labeled A, B, C, and D are shown in the corresponding panels A, B, C, and D. For ZE.5/I, panel A (mouse 134-2), DNA containing 4.1 kb of 5′ flanking DNA was prepared by purification of a Sma I–Cla I fragment as described in Fig. 1. Expression of ZE.5/II, panel B (mouse 188-1), was obtained from mice generated with a DNA fragment containing −5.3 to −3.8 kb of 5′ flanking DNA fused to ZE.5. Panel C depicts the results of expression of the ZE.5/III construct (mouse 193-1) bearing −6.6 to −5.3 kb fused to ZE.5, and panel D represents the expression of the ZE.5/II+III, which contains −6.6 to −3.8 kb of 5′ flanking DNA (mouse 151-2). Each of the fragments for deletion analysis was prepared from a clone such that the only vector DNA included is the 25 bp from the Eco RI to the Cla I site of pBR322.

including the continued methylation of critical regulatory regions or the presence of specific chromosomal proteins.

It is possible that structural elements capable of limiting the accessibility of the gene reside within the endogenous AFP locus but not in the constructions tested. The albumin and AFP genes have remained linked in both mice and humans through 300 to 500 million years since they arose from a common ancestor, with the albumin gene 13.5 kb upstream of the AFP gene and in the same transcriptional orientation (*28, 29*). To eliminate the possibility that modulatory elements are contained in the conserved intergenic region not contained within ZE, the entire 14-kb region between the 3′ end of the albumin gene and the 5′ end of the AFP gene was introduced into mice along with the minigene (YZE, Table 1). Studies in stably transfected F9 teratocarcinoma cells (*30*) had previously shown that constructs containing the entire intergenic region were activated upon differentiation to visceral endoderm, although in general at low levels relative to the endogenous mRNA. The YZE construct gives high levels of expression in all three tissues (Table 1), similar to that of ZE (Fig. 1). Thus, if the exceptionally high levels of minigene expression are due to the absence of a repressor the element lies outside of the intergenic region. Such an element could very well lie elsewhere, such as in the intragenic region deleted in the minigene, or the 3′ flanking region. A particularly intriguing possibility is that modulation requires the transcription of the 5′ albumin gene.

Developmental regulation of the AFP minigenes in liver and gut. The AFP mRNA declines rapidly after birth in the mouse liver and gut (*31*). This developmental regulation in liver is achieved as a result of a decrease in transcription (*31*) and is under the influence of the *raf* gene (*8*), an unlinked locus that acts in a cell-autonomous manner (*32*). In the gut, on the other hand, the repression is not *raf*-dependent (*9*). To determine whether there is a specific DNA sequence required for repression and whether the signals in liver and gut are functionally distinct, we examined the progeny of founder mice carrying ZE, ZE.5/I, and ZE.5/II+III minigenes.

The appropriate developmental pattern of AFP expression in both liver and gut was maintained in four out of five independent lines of ZE-bearing transgenic mice (Fig. 3). The level of minigene mRNA decreased by several orders of magnitude between days 1 and 14, to the same extent as the endogenous AFP mRNA. Thus, the signals for complete extinction in both liver and gut are contained within the minigene, promoter, and three enhancer domains. This is consistent with previous experiments where transcripts in mice carrying ZE plasmids were shown to decline in liver (*16*). However, those two lineages were both expressing the minigene at less than 10 percent of the endogenous level; therefore, the levels in the gut were exceedingly low and the completeness of extinction in both tissues could not be assessed. Complete postnatal transcriptional repression of the AFP minigene in liver and gut of transgenic mice can also be directed by elements I and II+III alone (Fig. 3). The fact that ZE, ZE.5/I, and ZE.5/II+III can all be developmentally regulated argues that no single enhancer is directing the postnatal repression in liver. Whether there is redundancy in the shut-off signals for both liver and gut in the enhancers themselves, or whether the repression signals are elsewhere in the gene, such as in the tissue-specific promoter region, remains to be determined by testing further constructs.

There were several examples where repression was incomplete, and was presumably affected by the chromosomal position. For example, in one ZE line (166-2), and one ZE.5/II+III line (160-2A), the minigene mRNA was incompletely shut off in liver while the levels of authentic AFP mRNA decreased appropriately. In animals bearing the ZE.5/I construct, incomplete suppression of AFP expression in one of three lineages occurred in the gastrointestinal tract, but not liver (mouse lineage 176-1, Fig. 3). This result implies either that there is a difference in the mechanism of repression in liver and gut or that transcription in the two tissues responds differentially to the same chromosome locations.

It is unknown at present whether repression is accomplished by negative regulation via a specific repressor or the disappearance of positive transcription factors, or both. The high frequency with which we observed persistent expression of the minigene into adulthood is difficult to reconcile with a model for extinction in which there is a rapid loss of gene-specific transcription factors after birth. We cannot rule out the possibility that some integrated copies have been mutated or rearranged, and thus become deregulated. Nor can we exclude the possibility that persistent lines result from the fortuitous integration of the fragments near strong cellular

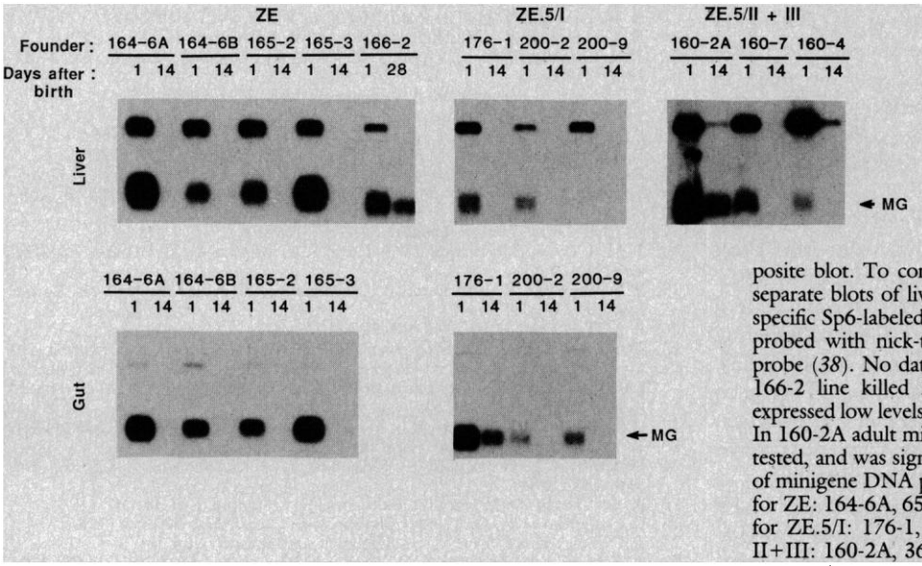


Fig. 3. Developmental regulation of the AFP minigene under the control of different combinations of distal enhancer elements. Progeny generated from mating founder mice bearing the constructs ZE, ZE.5/I, or ZE.5/II+III to C57BL/6 × SJL/J F_1 hybrids were killed and analyzed at days 1 and 14 after birth. Transgenic animals were identified by Southern blotting of liver DNA and poly(A)$^+$ RNA was analyzed as described in the legend to Fig. 1. The data from representative animals of each litter are presented in this composite blot. To control for quantity and integrity of mRNA loaded, separate blots of liver mRNA's were hybridized to a mouse albumin-specific Sp6-labeled probe (exon 1) (*2*) and blots of gut mRNA's were probed with nick-translated or Sp6-labeled mouse α-action mRNA probe (*38*). No data were available on gut samples of progeny of the 166-2 line killed 28 days after birth. All three ZE.5/II+III lines expressed low levels of AFP minigene mRNA in gut at 1 day after birth. In 160-2A adult mice, the level of the minigene mRNA in the gut was tested, and was significantly lower than at birth. The number of copies of minigene DNA per cell in each animal was approximately as follows for ZE: 164-6A, 65; 164-6B, 16; 165-2, 32; 165-3, 58; and 166-2, 58; for ZE.5/I: 176-1, 11; 200-2, ~100; and 200-9, 12; and for ZE.5/II+III: 160-2A, 36; 160-7, 3; and 160-4, 11. MG, minigene.

enhancers. What our results do say is that the mechanism of repression is apparently very sensitive to chromosomal location, far more so than the establishment of expression during development.

The reinduction of gene expression. The synthesis of AFP can be transiently reactivated in liver cells during the period of DNA synthesis that accompanies liver regeneration. The degree of inducibility in the adult liver is strain-dependent and under the influence of an unlinked, autosomal-dominant gene termed *Rif* for regulation of induction of AFP (*8*). To determine what regulatory elements are required for induction of AFP expression in adult liver, we used carbon tetrachloride to induce liver damage in 4-week-old F_1 mice carrying ZE, ZE.5/I, or ZE.5/II+III constructs. The animals were killed 3 days after treatment, and poly(A)$^+$ RNA was isolated and analyzed for AFP and minigene mRNA. In four independent lines of ZE mice and in single lines containing element I or element II+III, AFP minigene expression was induced during liver regeneration (Fig. 4). With line 160-2A, induction could be observed above the background level of persistent expression. In general, the level of minigene mRNA relative to the endogenous mRNA in the induced livers was equivalent to that observed in the same lines at birth. One exception to this was line 176-1, where induction was considerably greater during liver regeneration than that at birth (compare Figs. 3 and 4). Thus, elements I alone, II and III together, or all three can direct induction of AFP in the livers of transgenic mice in a variety of chromosomal locations. The regulatory elements required for the reinitiation of expression in adults remain accessible and are probably coincident with those required for the high level expression in utero.

Multiple enhancers in combination influence extent of expression. The experiments described above allow us to draw several conclusions regarding the mechanisms by which the mouse AFP gene is activated and modulated during development.

1) The AFP gene requires the presence of at least one of the three enhancer domains for the establishment of its transcription in the visceral endoderm, fetal liver, and gut. This is an unequivocal demonstration of a requirement for a cellular enhancer for the activation of gene expression during mammalian differentiation. Whether the enhancers are necessary to maintain expression remains to be established.

2) The presence or cotranscription of the tightly linked albumin gene is not required for the establishment of AFP transcription during development nor its modulation after birth. The linkage of the albumin and AFP genes in both mice and humans has survived 300 to 500 million years in evolution (*28, 29*). This fact, together with the observations that the genes are temporally coactivated and coexpressed in all three cell lineages, suggested that they might share regulatory elements involved in the activation of gene expression (*31*). It remains possible that the large enhancer domain in the intergenic region affects albumin gene transcription. This can be directly tested by appropriate constructs in transgenic mice.

3) The tissue-specific diversity in expression of endogenous AFP mRNA is not simply due to different concentrations of limiting trans-acting factors in each tissue because the expression of the AFP minigene in ZE-bearing transgenic mice was high in all three tissues. Rather, accessibility of the gene to trans-acting factors must play a significant role in the generation of tissue-specific diversity.

The possibility that the multiple enhancers act in a combinatorial fashion to generate the different levels of expression of the gene is suggested from their very different strengths in vivo. This conclusion, however, must be tempered by several considerations. First, most of the transgenic lines we examined contained multiple copies of the minigenes, but the correlation between copy number and expression was poor. Therefore, it is difficult to calculate the efficiency of transcription per gene copy. Second, all the constructs exhibited some position effects (Tables 1 and 2), although they were

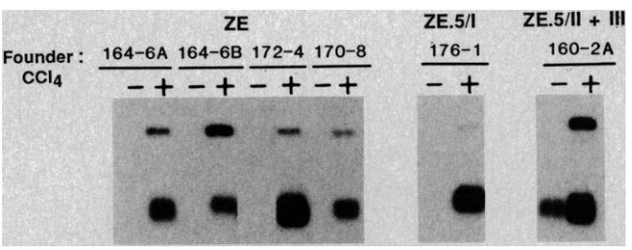


Fig. 4. Reinduction of the AFP minigenes during liver regeneration. Founder mice or F_1 males containing the vector-free DNA fragments of ZE, ZE.5/I, and ZE.5/II+III were mated to BALB/cJ mice, and transgenic animals were identified by Southern blots of tail DNA. Transgenic animals were injected with 100 μl of mineral oil (−) or a freshly prepared, 10 percent CCl_4 in mineral oil emulsion (+) on day 28 after birth and killed 72 hours after injection (*8*). Poly(A)$^+$ mRNA was prepared from frozen livers and analyzed by Northern blotting with the use of an Sp6-labeled AFP exon 1–specific probe (*16*). Typical animals from each litter are shown here. Separate blots were probed for albumin mRNA to control for quality and amounts of mRNA loaded. The copy number of the minigene was ~100 for 172-4 and 55 for 170-8. The copy numbers for the others are listed in the legend to Fig. 3.

minimized when all three domains were present (Fig. 1). The higher susceptibility of the smaller constructs to the site of integration suggests that all three enhancers are required for the gene to establish efficient transcription, at least in heterologous sites. The problems of copy number and position effects are partially compensated by our large survey of more than 50 different transgenic animals.

Apart from these reservations, the data in Fig. 2 and in Table 2 show that each of the three enhancers individually can direct expression to all three tissues but do not have equivalent biological activities as we tested them. This is unlike the tissue-specific pattern of *Drosophila* yolk protein gene expression where different cis-acting elements are active exclusively in either fat bodies or ovaries (*10, 33*). We favor a model whereby the accessibility of the AFP gene to transcription factors in conjunction with differences in enhancer recognition efficiency is responsible for the very different levels of expression of the endogenous gene in yolk sac, liver, and gut.

The application of this approach to studying the initiation of AFP gene expression in the fetus has given us insight into the mechanisms for generating diverse levels of expression in several tissues. Furthermore, the ability of the transgene to be modulated in newborns and adults demonstrates the feasibility of mapping the responsive elements in vivo.

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