Differential requirements for cellular enhancers in stem and differentiated cells

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ABSTRACT

Multiple cis-acting regulatory elements consisting of three cellular enhancers and a proximal promoter element have been identified in the region upstream of the mouse α -fetoprotein (AFP) gene. We examined the role of these sequences during differentiation by the introduction of modified AFP genes into cells at different stages of commitment to its expression. Modified AFP genes introduced stably into F9 embryonal carcinoma stem cells by DNA transfection were silent until activated by treatment with retinoic acid to form visceral endoderm. Their activation required the presence of both the enhancer and proximal promoter domains. The introduced genes activated simultaneously with the endogenous AFP genes, but reached maximal levels of expression more rapidly, suggesting a greater initial accessibility to transcription factors. In contrast, when modified AFP genes were stably introduced into HepG2 cells, a human hepatoma cell line that constitutively expresses the AFP gene, the proximal promoter sequences were sufficient to direct a low level of expression. The absolute requirement for the AFP enhancers in F9 cells but not in HepG2 cells supports a model by which there is an obligate requirement for enhancers during differentiation in addition to their role in enhancing gene expression after differentiation.

INTRODUCTION

The demonstration in the last several years that tissue-specific genes are regulated by multiple cis-acting elements (1-6) has raised the issue of the role of these elements in the initial activation of genes in stem cells and their modulation in the differentiated derivatives. Two classes of positive regulatory elements have been observed for RNA polymerase II genes; enhancers which are by definition position and orientation independent and can function on heterologous promoters (7) and promoter-proximal elements which are orientation but not position independent (8,9) and are usually positioned within 100 base pairs of the initiation of transcription. In general the functions of these elements have been assayed in fully committed cells, thereby leaving unresolved their role during differentiation.

We have begun to address the role of enhancers and promoters during differentiation by introducing the mouse α -fetoprotein (AFP) gene into both stem and differentiated cells. This gene, along with the closely linked and evolutionarily related gene coding for serum albumin are co-activated in the visceral endoderm of the yolk sac, the fetal liver and gut (10). AFP gene transcription is selectively shut down in neonatal liver, whereas expression of both genes is lost in the neonatal gut (11).

In experiments utilizing the transient introduction of AFP genes into human hepatoma cells, four regulatory elements have been identified; three cellular enhancers are positioned between the two genes at -6.5, -5.0 and -2.5 kilobases (kb) of DNA relative to the start of AFP gene transcription and a promoter-proximal element maps between -118 and -33 base pairs (bp) of DNA (4,46).

At least one of the three enhancers has been shown to be absolutely required for expression of the AFP gene in transgenic mice, in that an internally deleted AFP minigene construct lacking all three enhancers was not expressed in any tissue at any stage in development (12). In contrast, the identical construction was expressed at a low but easily detectable level in transient expression assays in hepatoma cells, and the effect of adding the enhancers was a 20 increase in the transcription of the minigene. This difference suggested to us the possibility that enhancers might have two different roles; one during differentiation in stem cells which involved the initial activation of the gene, and a subsequent quantitative one in differentiated cells.

To investigate this possibility further, we decided to look at the requirement for the AFP enhancers in another differentiating system, the F9 embryonal carcinoma (EC) cell line (13,14). Upon treatment with retinoic acid and cyclic AMP F9 cells differentiate to a cell closely resembling parietal endoderm (15,16). If the same cells are treated with retinoic acid under conditions promoting aggregation most of the cells on the outer surface of the aggregate acquire the phenotype of visceral endoderm, as demonstrated by *de novo* synthesis of AFP (17).

Our findings demonstrate that the enhancers are essential for the activation of the AFP gene in visceral endoderm during F9 cell differentiation, in complete agreement with our results in transgenic mice. To ask whether the activity of the AFP promoter alone in transient expression assays is a function of the non-chromosomal state of the gene, we also examined the requirement for the enhancers in stably transformed HepG2 cells, a differentiated liver-derived cell line which expresses the AFP gene constitutively. These studies support the model whereby the enhancers have an obligate role during gene activation, and a quantitative role thereafter.

MATERIALS AND METHODS

Vector and Constructions

The AFP minigene, ZE, was constructed by ligating together two previously subcloned Eco RI genomic fragments of the AFP gene (Z and E;19,20) producing a five exon minigene containing the first three and last two exons of AFP in addition to 7.6 kb of 5' flanking DNA. pZE.5 is a derivative of ZE, truncated at -1 kb in the 5' flanking region by digesting pZE with Bam HI followed by religation (4). In pZE Δ 8 an internal deletion of the 5' flanking sequence of pZE removes the sequences between -800 and -33 bp. This construction was generated in two steps. The first step involved adding an Xba I linker to an artificial Bam HI site at -33 bp in the minigene (the Δ 8 construction in ref. 38). To this was ligated an Xba I-Eco RI fragment which contains the 5' flanking domain between -800 to -7600 bp.

A plasmid containing the 3.4 kb Bam HI fragment of the Herpes simplex virus thymidine kinase gene (HSV tk) subcloned into pBR322 (ptk) (21) was used as a selectable marker for co-transfection of F9 tk⁻ cells. For linked transfections, Sal I linkers were added to the 2.0 kb Pvu II fragment of the HSV tk gene and this was ligated into the unique Sal I restriction site of the pBR322 vector portion of the AFP minigene plasmids. In pZE/TK1 both transcription units share the same clockwise transcriptional orientation. In pZE/TK2 the AFP transcriptional orientation is clockwise

while the tk transcriptional orientation is counterclockwise, and in pZE/TK4 the AFP transcriptional orientation is counterclockwise while the tk transcriptional orientation is clockwise. Positive selection for uptake by Hep G2 cells with either pZE or pZE.5 was achieved by co-transfection with either pSV₂neo or with pSV232Aneo, a derivative of pSV₂neo lacking the 72 bp repeat enhancer sequence, a generous gift of Dr. Tom Kadesch (22).

Cell Culture and Transfection

F9 tk⁻ cells, obtained from Davor Solter and Barbara Knowles, were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal calf serum (FCS). Stable transformants of F9 EC cells were generated by DNA mediated transfection in the presence of Ca₃(PO₄)₂ and identified by HAT selection as described previously (20).

Differentiation of the EC cells to either parietal endoderm-like cells or visceral endoderm-like cells was as described previously (20).

Hep G2 cells obtained from B. Knowles were maintained in medium composed of a 1:1 mixture of DMEM and Ham's F12 supplemented with 10% FCS and 10 µg/ml bovine pancreas insulin (Sigma). To obtain stable transformants of Hep G2 cells, 15 µg of DNA (12 µg of AFP plasmid and 3 µg of the selectable marker plasmid) were precipitated in Ca₃(PO₄)₂ and applied to approximately 4x10⁶ cells per 100mm culture dish. After 16 hr the medium was withdrawn and the cultures were refed with 10 ml of fresh medium. Approximately 24 h later the cultures were refed with selective medium containing either 600 µg/ml (pSV₂neo) or 400 µg/ml (pSV232Aneo) of G418 (Geneticin, GIBCO). After the appearance of G418-resistant colonies (approximately two weeks), the level of G418 in the medium was reduced to 400 µg/ml in all cases. After three weeks G418^R colonies were picked and expanded for DNA analysis. Cultures expanded for analysis of AFP minigene expression were maintained in nonselective medium.

DNA Analysis

Individual F9 HAT^R and Hep G2 G418^R clones were expanded and genomic DNA isolated as described previously (20). Total genomic DNA was digested with appropriate restriction enzymes that permitted the introduced minigenes to be distinguished from the endogenous AFP gene. The digested fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose by the method of Southern (23). AFP-specific sequences were detected by hybridization to one of two unique copy gel-purified fragments: a 440 bp Hinc II fragment that spans the first exon of the mouse AFP gene or a 2100 bp Hha I-Eco RI fragment that extends from the intron between exons 1 and 2 to within the intron between exons 3 and 4. A 1.34 kb Hind III-Nru I fragment from pSV₂neo was used to detect neo gene sequences. Filters were hybridized at 42°C in 50% formamide containing dextran sulfate and processed as described previously (24). The DNA copy number of minigenes was based on densitometric scanning of autoradiographs assuming the endogenous AFP signal represents 2 copies per cell.

RNA Analysis

Total cellular RNA was prepared by the hot phenol method (25) and enriched for polyadenylated sequences by oligo (dT)-cellulose chromatography (26). Poly (A)⁺ RNA size-separated by electrophoresis through 1.5% agarose containing 6% formaldehyde in MOPS buffer (20mM morpholinepropanesulphonic acid, 5mM sodium acetate, 1mM EDTA; pH 7.0) was transferred directly to nitrocellulose in 20x SSC (1x SSC = 0.15M NaCl, 0.015M Na citrate) (27). The filters were hybridized to a SP6-generated single stranded first exon RNA probe as described previously (28).

RESULTS

The enhancer domain between -7.6 kb and -1 kb is essential for tissue specific activation of the AFP gene in visceral endoderm. We had

previously demonstrated that an AFP gene construct containing the entire 14 kb of intergenic DNA between the 3' end of the albumin gene and the 5' start of the AFP gene was activated following retinoic acid treatment of stably transfected F9 stem cells in a tissue specific manner (20). To test whether this activation could occur in the absence of the 3 AFP gene enhancers, located between -1 and -7.6 kb, relative to the start of transcription at +1, the appropriate flanking DNA sequences were fused to an AFP minigene, composed of the first three exons and the last two exons of the AFP gene along with 350 bp of 3' flanking sequence (see Fig 1). Transcription and processing of the AFP minigene gives rise to a 600 nucleotide polyadenylated RNA easily distinguished from the 2.2 kb endogenous AFP mRNA by gel electrophoresis (20).

To generate stable DNA transformants recipient F9 EC cells deficient for thymidine kinase (tk^-) were transfected with AFP minigenes which contained the enhancer domain (ZE) or was missing it (ZE.5) along with the Herpes virus thymidine kinase (tk) gene, followed by selection in HAT medium. Linkage of the two genes was employed in some instances to increase the very low co-transformation efficiency previously observed (~15%; 20). The linkage of the tk gene made no difference in the outcome of the experiments, except to increase the rate of co-transformation.

Of the 85 HAT^R ZE clones identified by genomic DNA analysis with AFGP-specific probes which could distinguish between the endogenous and exogenous genes, 36 (42%) contained the AFP minigene. These clones were then analyzed for mRNA expression in the stem cells and in differentiated parietal and visceral endoderm derivatives by gel electrophoresis of poly A⁺ RNA followed by blotting and hybridization to an AFP fragment as described by Scott et al. (20).

Twelve of twenty-two individual clones and two pools containing the ZE minigene expressed AFP minigene RNA in a qualitatively identical manner

FIG. 1 The Structure of the AFP Constructs. The first line in the diagram represents the positions of the albumin and AFP genes on chromosome 5, separated by 14 kb of DNA. In the second line are shown the positions of the three enhancer elements, labelled I, II and III in the stippled area, the promoter-proximal domain, depicted as a thick black line, and the derivation of the first three and last two exons of the minigene body, indicated by the black boxes. In the following lines are the portions of the locus which are contained within the constructs used in the study. YZE, ZE and ZE.5 are 5' truncations of the flanking region at -14, -7.6 and -1.0 kb, respectively. ZEΔ8 has the promoter-proximal region between -800 and -33 deleted as indicated.

to the endogenous AFP locus (Table 1 and Fig. 2). Expression of the minigene RNA was never observed in either stem cells or in parietal endoderm derivatives, although in some cases aberrant transcription occurred in both as seen previously (20). For example, clone ZE-20 exhibited a single transcript larger than 600 nt in all three cell types (Fig. 2). This aberrant transcript was shown to have initiated upstream of the correct site by S1 nuclease analysis (data not shown). In clone ZE-18 both aberrant expression and proper expression were observed. Whether both were occurring in the visceral endoderm derivatives could not be determined because the aggregates contain a substantial number of non-visceral endoderm cells (29). These results, which are consistent with our previous study in which 14 kb of 5' flanking DNA was utilized, suggest that there is no contribution from the sequences between -14 kb and -7 kb to either tissue specific repression in stem cells and parietal endoderm or to tissue specific expression in visceral endoderm. This finding is also consistent with our inability to assign any functional role to these sequences as assayed by AFP minigene RNA expression in tissues of transgenic mice or in transient expression in Hep G2 cells (4,12,28).

The proper activation of the minigene was coincident with the activation of the endogenous AFP gene at day 6 as shown in Fig. 3, although the minigene mRNA reached a maximal level more rapidly than the endogenous mRNA.

The F9 stable transformants generally expressed the introduced AFP gene at low levels relative to the endogenous locus and only in high copy number transformants (Table 1). It may be relevant that similar low levels were observed in initial studies of AFP gene expression in transgenic mice (28), a problem which was overcome by the removal of plasmid sequences before microinjection (12). By extrapolation the inability to achieve high levels in F9 derived visceral endoderm may be due to the presence of linked plasmid DNA sequences.

We next examined the ZE.5 stable transformants to ask whether the sequences between -7 kb and -1 kb which contained the three cellular enhancers defined by transient expression assays (4) were required for the tissue specific activation of the gene. None of the 16 stable clones analyzed expressed the AFP minigene in either F9 stem cells, visceral endoderm, or parietal endoderm (Table 1 and Fig. 2). It should be emphasized here that had the ZE.5 minigene been activated to 1% of the highest level observed in the ZE transformants, the assay was sufficiently sensitive to detect it. That is, the removal of the enhancers must have a greater than 100-fold effect on the activity of the gene. These results demonstrated a functional requirement for sequences between -7 kb and -1 kb in gene activation. A similar requirement for at least one of the enhancers has recently been demonstrated in transgenic mice (12).

The DNA between -800 bp and -33 bp is essential for tissue specific activation of the AFP gene in visceral endoderm. Previous analysis by transient expression of AFP sequences into HepG2 cells had identified a second region between -118 and -33 bp critical to the expression of the gene (4), although this same region was not required for expression in HeLa cells in the presence of the SV40 enhancer (30). To test for the requirement of this region for gene activation, an AFP construct containing all three enhancers but with the sequences between -800 bp and -33 bp deleted was placed 5' of the AFP minigene and then linked to the HSV tk gene (pZEA8) (Fig. 1). Following transfection into F9 tk- cells, individual transformants were isolated and analyzed for their ability to activate the minigene (Table 1 and Fig. 2, lanes 16 and 17). None of 16 clones analyzed exhibited proper expression of the AFP minigene. These results argue that the enhancer sequences are necessary but not sufficient for the activation of the gene, and that more proximal sequences are required.

Expression in HepG2 cells is not enhancer dependent but is enhancer responsive. The experiments in both F9 cells and transgenic mice (12) strongly argue that the enhancer domain of the AFP gene was necessary to effect the selective activation of the AFP minigene. In contrast, in

TABLE 1. Expression of AFP minigenes in F9 stable transformants.

CONSTRUCT	CLONE	DNA COPY # ^a	MINIGENE EXPRESSION ^b		
			S	V	P
ZE/TK1	1	1-5	-	-	n.a.
	2	1-5	-	-	-
	3	1-5	-	+	-
	4	1-5	-	+	-,*
	5	5-15	-	-	-
	6	25-35	-	-	-
	7	100-120	-	-	-
	8	100-120	-	+	-
ZE/TK2	9	1-5	-	-	n.a.
	10	1-5	-	-	-
	11	1-5	-	-	-
	12	1-5	-	-	-
	13	40-60	-,*	-,*	-,*
	14	80-100	-	+	-
	15	80-100	-	+	-
	16	80-100	-	+	-
	17	100-120	-	+	-
	18	100-120	-,*	-,*	-,*
	19	120-140	-	+	-
	20	120-140	-,*	-,*	-,*
ZE/TK4	21	1-5	-	-	-
	22	10-20	-	+	-
	23	60-80	-	+	-
	24	60-80	-,*	+,*	-,*
ZE.5/TK1	1	1-5	-	-	n.a.
	2	1-5	-	-	n.a.
	3	1-5	-	-	n.a.
	4	10-20	-	-	n.a.
	5	20-30	-	-	-
	6	25-35	-,*	-,*	-,*
	7	25-35	-	-	n.a.
	8	60-80	-	-,*	-,*
	9	120-140	-	-	-
	10	200-250	-,*	-,*	-,*
ZE.5/TK2	11	1-5	-	-	n.a.
	12	1-5	-	-	n.a.
	13	1-5	-	-,*	n.a.
	14	15-25	-,*	-,*	n.a.
	15	120-140	-	-	-
	16	140-160	-	-	-

ZE48	1	1-5	-	-	n.a.
	2	1-5	-	-	n.a.
	3	1-5	-	-	n.a.
	4	1-5	-	-	n.a.
	5	1-5	-	-	n.a.
	6	1-5	-	-	n.a.
	7	1-5	-	-	n.a.
	8	1-5	-	-	n.a.
	9	10-20	-	-	n.a.
	10	15-25	-	-	n.a.
	11	120-140	-,*	-,*	n.a.
	12	140-160	-,*	-,*	n.a.
	13	140-160	-,*	-,*	n.a.
	14	140-160	-	-	n.a.
	15	140-160	-	-	n.a.
	16	200-250	-,*	-,*	n.a.

^a DNA copy number was estimated from autoradiographs by densitometric analysis of Southern blots using the endogenous AFP locus as an internal standard for 2 copies per cell.

^b AFP minigene expression was determined for S(F9 EC cells); V(embryoid body visceral endoderm derivatives); and P(parietal endoderm derivatives) by northern blotting as shown for representative examples in Figure 2. The symbol (-) represents the absence of AFP minigene RNA; (+) the presence of bona fide AFP minigene RNA; and (*) the presence of an aberrant AFP RNA. N.a. stands for not analyzed.

transient assays in HepG2 cells minigenes without the enhancers were able to direct a low but significant amount of minigene expression. This level was increased 30-fold by the addition of one, two or all three of the enhancer elements (4). It was possible that this apparent differential requirement for the enhancers between F9 cells and HepG2 cells could be attributed to a difference in the nature of the two assays (transient vs. stable). To examine this possibility we created individual stable transformants of HepG2 cells containing the AFP minigene with and without the enhancer domain (ZE and ZE.5, respectively, see Fig. 1).

HepG2 cells were co-transfected with the AFP constructs along with the dominant selectable marker, pSV₂neo. Clones were selected on the basis of resistance to the antibiotic G418 and subsequently analyzed for the presence of the AFP minigenes by genomic Southern analysis. Representative AFP positive clones analyzed for expression from the exogenous AFP genes by Northern gel blotting are shown in Figure 4. There was some clonal variation in AFP minigene expression but strikingly, all three clones without the enhancers (ZE.5) showed comparably high levels of expression when compared to the clones with the enhancers (Table 2 and Fig. 4A). This result stands in contrast with those in F9 cells where no expression was ever detected from ZE.5. Furthermore whereas F9 transformants required the integration of multiple copies of the minigene for expression single copy transformants were expressed at high levels in HepG2 cells (Table 2).

The apparent absence of an effect of the enhancers in HepG2 cells was not predicted by the transient expression studies, where the enhancers had a ~30-fold enhancing effect on transcription from ZE.5. This paradox led us to suspect that the SV40 enhancer in the co-transfected pSV₂neo plasmid might be acting in *cis* at the sites of integration of the exogenous DNA to affect ZE.5 gene expression.

FIG. 2. AFP minigene RNA expression in representative F9 stable transformants before and after differentiation. Total poly(A)⁺ RNA (7μg per lane) from F9 stable transformants isolated from untreated F9 cells (S, stem), or from cells treated for 5 days as monolayers with 5×10^{-8} M retinoic acid and 1×10^{-3} M dibutyryl cyclic AMP (P, parietal), or from cells treated with 5×10^{-8} M as aggregates for 3 days and maintained as aggregates for a total of 14 days to form embryoid bodies (visceral, V) was fractionated by electrophoresis through a denaturing formaldehyde gel (1.7%) and transferred to a nitrocellulose filter. AFP RNA was detected by hybridization of the filter with a ³²-P labelled single stranded RNA probe from the first exon of AFP. The lane marked M contains fetal liver poly (A)⁺ RNA and poly (A)⁺ from transfected Hela cells as standards for the migration of authentic 2.2 kb AFP mRNA and 0.7 kb minigene RNA. Lanes 1-17 correspond to RNA from the indicated individual F9 stable transformants ZE-17, ZE-18, ZE-20, ZE-23, ZE.5-9, and ZEΔ-8-4 containing the constructs described in Fig. 1 as indicated.

To eliminate this potential complication, pSV232Aneo, a plasmid from which the two SV40 72 bp repeats were removed (22), was co-transfected with ZE and ZE.5 as described above. The resulting G418 resistant cells were analyzed as before. In contrast to the results obtained with pSV₂neo, on average there was a 30-fold difference between the levels of minigene expression between ZE and ZE.5 transformants (Table 2 and Fig. 4B). This difference is very similar to that observed in the transient expression assays (4), and argues that the expression of the AFP minigene in the absence of the enhancers in HepG2 cells cannot be attributed to the absence of integration of the plasmid into the host chromosome. Rather these results suggest that the ability of ZE.5 to be expressed in hepatoma cells but not in F9 cells or tissues of transgenic mice is not a function of the assay conditions, but appears to reflect the nature of the recipient cell.

DISCUSSION

The absolute requirement for the enhancer domain in the activation of the AFP gene during the formation of visceral endoderm is consistent with experiments in transgenic mice, where the enhancers were essential for activation of the gene in three cell lineages, the visceral endoderm, liver

FIG. 3. Time course of the activation of endogenous AFP mRNA and AFP minigene RNA during the differentiation of F9 stem cells to visceral endoderm. The lane marked M is a standard for the migration of minigene RNA. The migrations of authentic AFP mRNA at 2.2 kb and minigene RNA at 0.7 kb are indicated. Total poly (A)⁺ RNA (7 μ g per lane) from the F9 stable transformant ZE-23 was processed as described in Fig. 2 for (S, stem), (P, parietal) and (V, visceral) cell types. The 4,6,9,12, and 14 in lanes 3-7 correspond to days after plating in retinoic acid and aggregation conditions. To control for RNA loading and transfer the filter was rehybridized with a cloned probe to a constitutive ribosomal protein RNA (data not shown).

and gut (12). In contrast, the presence of the enhancers was not required for activity of the AFP minigene when integrated stably into HepG2 cells. Rather their effects were quantitative; that is, they increased transcriptional activity of the AFP minigene approximately 30-fold. Our failure to detect AFP minigene transcripts in ZE.5 F9 transformants upon differentiation cannot be attributed to the sensitivity of the assay; had their quantitative effects been the same in F9 cells as they were in HepG2 cells, we would have detected the lower level of transcripts.

A second region between -800 and -33 bp was shown to be essential for the activation of the AFP gene. A segment of this region between -118 and -33 bp was previously shown to be required for transient expression of the AFP gene in HepG2 cells while having no activity in HeLa cells in the presence of the SV40 enhancer (4,30). It is likely that the absence of this region is responsible for the failure of the gene to activate in F9 cells.

We interpret the differential requirement for the enhancers for minigene expression in fertilized eggs and F9 cells versus HepG2 cells as a reflection of the difference in the commitment of the recipient cells to AFP gene expression. In the stem cells, the introduced genes are initially quiescent and require activation. In HepG2 cells, the gene is introduced into a cell which is capable of actively transcribing it from the outset. Thus an enhancer is apparently required for the gene to go from a quiescent to an active transcriptional state. Our interpretation is limited to a

FIG. 4. AFP minigene RNA expression in HepG2 stable transformants. 5 μ g of polyA⁺ RNA from stable transformants of HepG2 cells were electrophoresed through agarose gels, blotted onto nitrocellulose filters and hybridized to an AFP-specific probe. A. In lanes 1-19 are ZE transformants 1, 21, 20, 15, 19, 9 and 5 and ZE.5 transformants 2,3, and 1 generated by co-transfection with pSV₂neo as described in Table 2. B. In lanes 1-14 are ZE transformants 2, 3, 10, 9, 11, 12, 13, 5, 6, and 7 and ZE.5 transformants 5, 6, 4, and 2 generated by co-transfection with pSV232Aneo as described in Table 2. The last 4 lanes marked 11-14 are shown at 6 hr exposure (1X) and 180 hr exposure (30X).

certain extent by the fact that we are comparing the behavior of the AFP gene in quite different cells (human versus mouse; visceral endoderm versus liver). However the fact that we observe an identical 20 to 30-fold effect of the enhancers in assays which utilize primary fetal mouse hepatocytes (R. Godbout and S. Tilghman, unpublished results) argues that the differences cannot be attributed to a species difference. Likewise the complete absence of expression of the ZE.5 minigene in all tissues of transgenic mice suggest that tissue differences cannot explain the results.

It could be argued that the failure to express the ZE.5 minigene in differentiated F9 cells as compared to HepG2 cells may simply be the consequence of the lower concentration of promoter-specific positive transcription factors in the former cells. However this seems a less likely explanation for the failure of ZE.5 to be expressed in transgenic mice, especially given that the AFP gene is expressed at very high levels in both visceral endoderm and fetal liver.

The obligate requirement of an enhancer during the initial activation of a gene has been tested for only a small number of genes,

TABLE 2. Expression of AFP minigenes in HepG2 stable transformants.

CONSTRUCT	CLONE	AFP MINIGENE COPY NUMBER	MINIGENE EXPRESSION
pZE+pSV ₂ neo	1	1	+++
	2	1	++
	3	1	0
	4	1	0
	5	1	++++
	6	1	++++
	7	2	+++
	8	2	++++
	9	2	++++
	10	2	0
	11	5-7	+++
	12	5	++++
	13	5	+++
	14	7-10	0
	15	7-10	++++
	16	7-10	+
	17	8-10	++++
	18	10	++++
	19	>10	+++
	20	10-20	++++
	21	>20	++++
pZE.5+pSV ₂ neo	1	1	++
	2	2	++++
	3	2	++++
pZE+pSV232Aneo	1	1	0
	2	1	+++
	3	1	+
	4	1	0
	5	1	+++
	6	1	++
	7	1	++
	8	2	0
	9	2	+
	10	2	+
	11	3	+++
	12	5-10	++++
	13	>50	++++
pZE.5+pSV232Aneo	1	1	0
	2	1	1/2+
	3	1	1/2+
	4	2	1/2+
	5	3	+
	6	3-5	1/2+

primarily using transgenic mice, where a consistent pattern has not emerged. For example an element with characteristics of a cellular enhancer which resides 10 kb upstream of the mouse albumin gene has an enhancing effect in transgenic mice, but is not absolutely required (31). Likewise the 3' enhancer of the human β -globin gene generates a higher level of transcript when it is present on the transgene, but is not absolutely required for expression in erythroid cells (32). In contrast, the deletion of the enhancer from the mouse immunoglobulin heavy chain gene results in the complete absence of its expression in transgenic mice (R. Grosschedl and F. Costantini, personal communication). Clearly a more detailed cataloguing of the regulatory elements in these genes, which might reveal the presence of multiple enhancer-like elements, will be necessary to resolve these differences.

The activation of genes during differentiation is thought to involve multiple events, including the acquisition of positive transcription factors and the generation of a chromatin configuration around specific genes such that they are now accessible to the factors (33). In several other systems which have been examined in detail, transfected genes have been actively transcribed in the absence of transcription of the endogenous gene (1,34), suggesting that the acquisition of transcription factors can precede the change in chromatin structure required for gene activation. In F9 cells, on the other hand, there is no specific transcription of either the exogenous or endogenous AFP genes until differentiation, arguing that both events must occur.

It is unlikely that the obligate role of the enhancer is to facilitate a change in chromatin structure of the gene, as the introduced gene is already in an accessible conformation, as evidenced by the observation that transcription initiating upstream of the AFP cap site (30) is occurring across the locus even in the stem cells (see Fig. 2). Thus the requirement for the enhancer must involve either a role in the establishment of a stable transcription complex, or relieving a stem cell-specific repression. If the former is true, then one has to propose that there is a difference between the establishment of a stable transcription complex in stem cells undergoing differentiation and in differentiated cells like HepG2 cells, in which the AFP minigene without the enhancers is transcribed.

The extensive examination of retrovirus gene expression in EC cells and preimplantation embryos has led to the proposal that these cells may possess a largely irreversible repression system for the regulatory elements of these viruses. This is not a general mechanism for repression of genes introduced into F9 cells, as shown by the high level expression of a transfected human calcitonin gene in these cells (38). The exact nature of the block to retroviral gene expression has not been elucidated but both the enhancers in the LTR and DNA methylation have been implicated in achieving this state (39,40). Studies of papovavirus gene expression have also implicated enhancers in gene repression in EC cells, possibly through the action of a cellular adenovirus ELA-like protein (41-43). Finally, an example of negative *cis* regulation of MHC Class I gene expression in F9 cells as assayed by transient expression has been reported (44).

If negative regulation prevents AFP gene expression in F9 cells it cannot proceed via any of the sequences between the 3' end of the albumin gene and -1 kb of 5' flanking DNA. Deletion of this 13 kb region led to complete repression of the gene, not to its activation in F9 stem or parietal endoderm derivatives. Muglia and Rothman-Denes (45) have reported a negative control region in the rat AFP gene at -3.5 kb, but it cannot explain the effects we observe with ZE.5. It should be emphasized that our failure to detect negative *cis*-acting sequences in F9 cells may be compromised by the relatively large regions deleted, in that obligate

positive acting sequences could have been deleted as well. The co-localization of positive and negative regulatory elements in papovaviruses and the MHC Class I gene suggest that this is a possibility (41-44).

To explain the differential requirement for the enhancers in F9 cells versus HepG2 cells we suggest that stem cells, whether they be F9 cells or cells of the early embryo, have a mechanism for stably repressing specific genes, and that one critical role for enhancers is to reverse that repression. In the case of the AFP gene, repression would proceed via DNA sequences within the gene itself or the proximal 1 kb of 5' flanking DNA. This repression is stem cell-specific and therefore is not operative in stable HepG2 transformants. Consequently a basal level transcription complex can be formed. The presence of the enhancers in HepG2 cells acts to modify the transcription complex to allow for more efficient transcription. This model makes the prediction that the deletion of the repressor domain of the gene, if it does not coincide with a positive regulatory domain, should permit basal level expression of the AFP gene upon differentiation even in the absence of the enhancers. Studies to test this model are in progress.

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