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A Globin Enhancer Acts by Increasing the Proportion of Erythrocytes Expressing a Linked Transgene

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Enhancer elements have been shown to affect the probability of a gene establishing an active transcriptional state and suppress the silencing of reporter genes in cell lines, but their effect in transgenic mice has been obscured by the use of assays that do not assess expression on a cell-by-cell basis. We have examined the effect of a globin enhancer on the variegation of *lacZ* expression in erythrocytes of transgenic mice. Mice carrying *lacZ* driven by the α-globin promoter exhibit β-galactosidase (β-Gal) expression in only a very small proportion of embryonic erythrocytes. When the transgenic construct also contains the αHS-40 enhancer, which controls expression of the α-globin gene, expression is seen in a high proportion of embryonic erythrocytes, although there are variations between transgenic lines which can be attributed to different sites of integration. Analysis of β-Gal expression levels suggests that expressing cells in lines carrying only the α-globin promoter express as much β-Gal as those in which the transgene also contains αHS-40. A marked decline in transgene expression occurs as mice age, which is mainly due to a decrease in the proportion of cells expressing the transgene. Thus, a globin enhancer can act to suppress variegation of a linked transgene; this result is consistent with a model in which enhancers act to establish and maintain an active domain without directly affecting the transcriptional rate.

The mechanism by which enhancers regulate gene expression remains in question. While enhancers have been widely considered as elements which act at a distance to increase the rate of transcription from a linked promoter (19, 42, 47, 51), several experiments that have assayed individual cells after gene transfer in cell lines have suggested that enhancers facilitate the establishment and maintenance of promoter activity with little or no effect on the level of gene expression in each cell (23, 24, 49, 50, 52). A recent study with cultured cells found that enhancers could retard silencing of an integrated reporter gene without significantly affecting the level at which it was expressed, suggesting that they function to disrupt or prevent the formation of repressive chromatin structures, thus permitting the maintenance of gene expression (50). Enhancers have frequently been used in transgenic mice to increase expression levels, and it has generally been assumed that this effect is achieved by directly increasing the rate of transcription of the transgene. The findings in cell lines suggested that this assumption might be incorrect and that instead the role of enhancers in transgene expression is to ensure gene expression in the appropriate lineages. A study of the effect of an enhancer on expression of a reporter gene in whole animals would provide evidence for or against this hypothesis, if expression of the reporter could be observed in single cells.

The human globin genes are found in two clusters; in each locus, they are arranged from 5′ to 3′ in the order in which they are expressed during ontogeny (5′ ζ-α2-α1 3′ in the α-globin locus and 5′ ε-^{G}γ-^{A}γ-δ-β 3′ in the β-globin locus) (44). The α-globin gene and its immediate flanking regions exhibit little or no expression in transgenic mice (12, 26, 36, 40), but a DNase I hypersensitive site 40 kb upstream of the ζ globin gene (αHS-40) confers high-level expression on linked globin genes in transgenic mice as assayed by RNase protection (16). αHS-40 acts as an enhancer in transient and stable expression assays in cell culture (29, 32, 41), and in common with other globin enhancers, is bound by a set of erythroid transcription factors (17, 45). All of the functional assays of the αHS-40 element described above were carried out with cell lysates, so that the nature of the enhancement αHS-40 conferred on gene expression was unclear.

We have recently reported variegated (heterocellular or mosaic) expression of a *lacZ* transgene driven by globin transcriptional control elements, including αHS-40 (33). This variegated expression is an "all or none" phenomenon; the gene is either active or completely inactive in an individual erythroid cell. Variegation has also been observed with a number of other transgenes; among them are tyrosinase (3, 22), myelin basic protein (18), hypoxanthine phosphoribosyltransferase (30), and human CD2 in T cells (7). Few studies have examined expression of globin transgenes in individual cells; when this has been done (by immunostaining with globin antibodies), variegated expression has been observed (8, 25, 43). Variegation is the visible result of a stochastic and clonally heritable silencing of gene expression. It appears to be the product of an equilibrium between repressive chromatin structures and active transcription units that produces a certain probability of stable repression (14, 46). Little information is available on the methylation status of genes silenced in this way. Variegation can be detected only when expression of a gene is examined on a cell-by-cell basis; when expression is studied in extracts from entire tissues, differences in the proportion of cells expressing a transgene will be seen as differences in total expression in that tissue.

In the study presented here, we have asked whether the αHS-40 enhancer element increases transcription by increasing the number of transcriptionally active cells (i.e. suppressing variegation in transgenic erythrocytes) or by increasing the level of expression in transcriptionally active cells. We have generated lines of mice carrying transgenes containing the *lacZ*

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gene driven by either the α-globin promoter alone or by the α-globin promoter with the αHS-40 element placed downstream of *lacZ*.

Histochemical staining for β-galactosidase (β-Gal) allows analysis of transgene expression in entire mouse embryos and at the single-cell level in erythrocytes; total β-Gal expression in erythrocytes can be assayed in blood lysates. β-Gal expression in erythrocytes is variegated in all lines. In lines carrying the construct containing only the α-globin promoter, extremely small numbers of erythrocytes strongly expressing β-Gal are seen in four of five lines. In lines carrying the construct containing both the promoter and the αHS-40 enhancer, high percentages of erythrocytes express β-Gal in the embryonic stage, but we find no evidence that the level of expression in these erythrocytes is higher than that in the few expressing erythrocytes in promoter-only lines. Greater silencing (fewer expressing erythrocytes) in some lines is not reflected in heavier methylation of the transgene promoter. In contrast, the transgene promoter in erythroid cells from a completely inactive line is heavily methylated. We also observe that as mice age, there is a decline in β-Gal expression which is mainly attributable to a decrease in the proportion of expressing erythrocytes. Our findings suggest that the αHS-40 enhancer acts to suppress the variegation of transgene expression in erythroid cells. This is consistent with the idea that enhancers function to create and maintain regions in which promoter activity is permitted.

MATERIALS AND METHODS

Constructs. The αP construct was made by fusing the human α-globin promoter from −570 to +40 to the SDK*lacZ* reporter gene, which encodes an *Escherichia coli* β-Gal expression cassette. The αPE construct was made by adding a 4-kb *Bam*HI fragment which contains the αHS-40 element to the 3′ end of the construct described above. For microinjection, both constructs were released from vector sequences with *Hin*dIII and *Kpn*I, and the fragments were purified by gel electrophoresis.

Production of transgenic mice. Transgenic mice were produced by microinjection of linearized DNA into the pronuclei of fertilized eggs from the outbred P.O. mouse strain, which were then transferred into psuedopregnant females. Transgenic progeny were identified, and hemizygous lines were established by mating transgenic founders to P.O. mice. Copy number was determined by standard Southern blot analysis of tail DNA. All data presented in this paper have been obtained from established transgenic lines.

Staining. For developmental studies, transgenic hemizygous males were mated to wild-type P.O. females, and the morning on which the copulatory plug was observed was considered 0.5 day postcoitum (dpc). Whole embryos were stained at 11.5 dpc. The embryos were dissected into phosphate-buffered saline (PBS), fixed, and washed for 1 h each and stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for 5 h at 37°C as previously described (33). To observe expression at the single-cell level, erythroid cells from mice at various time points were also stained with X-Gal. Embryos were bled into PBS, while blood from postnatal mice was collected by tail bleeding, and a dilute suspension of erythroid cells was fixed and washed for 5 min each and then was stained at 37°C for up to 24 h. A minimum of 200 erythroid cells from at least three positive embryos were counted, and the percentage of cells that stained blue with X-Gal was determined.

β-Gal assays. The activity of β-Gal in erythroid cells was assayed in lysates of blood obtained at various time points. Embryos were bled or adult peripheral blood was collected into 200 μl of PBS. A cell count from the blood of positive individuals was performed with a hemocytometer. The cells were collected by centrifugation, resuspended in 0.5 ml of ice-cold 250 mM Tris (pH 7.4), and lysed by four cycles of freeze-thawing, and the cellular debris was removed by centrifugation at 4°C for 10 min. Ten microliters of the lysate was assayed with the substrate *o*-nitrophenyl β-D-galactopyranoside (ONPG) as described previously (15) for 2 h, and A_{413} was determined. β-Gal activity values of lysates were calculated per erythroid cell.

Methylation of the transgene. Genomic DNA was extracted from erythroid cells of 12.5-dpc transgenic embryos of both αP and αPE lines and digested with *Pst*I and *Eco*RV. A 1.7-kb fragment containing the human α-globin promoter and the 5′ portion of the *lacZ* gene was detected when probed with the same *Pst*I-*Eco*RV fragment in Southern blot analysis. Some DNA was further digested with the methylation-sensitive restriction endonuclease *Eag*I or *Sma*I to test for methylation at those sites in the α-globin promoter. *Eag*I cuts at −54 relative to the transcription start site, and *Sma*I cuts at −86, −116, and −237. Four micrograms of DNA was digested with 30 U of each restriction enzyme for 16 h under the conditions described by the manufacturer. After electrophoresis, the agarose gel was stained with ethidium bromide and checked for equivalent DNA digestion by each enzyme in the samples before Southern transfer.

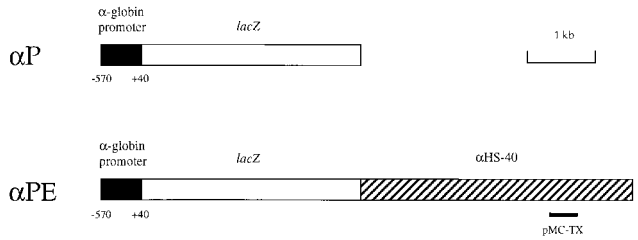

FIG. 1. αP and αPE constructs microinjected into fertilized mouse eggs to generate transgenic lines. The αP construct consists of 570 bp of the human α1-globin promoter linked to a 3.3-kb fragment which includes an *E. coli* β-Gal expression cassette. The αPE construct has, in addition to the former, a 4-kb fragment encompassing αHS-40 linked on the 3′ end. The bar denoted pMC-TX shows the position of the core 350-bp region which contains the major activity of αHS-40 as defined by Jarman et al. (17).

RESULTS

Expression and tissue specificity of the *lacZ* transgene. Two constructs were injected into fertilized mouse eggs to produce transgenic mouse lines (Fig. 1). αP contains 570 bp of the human α1-globin promoter linked to the *lacZ* reporter gene which encodes β-Gal. In αPE, a 4-kb *Bam*HI fragment containing αHS-40 has been added to the 3′ end of αP. In head-to-tail concatemers of the transgene, the αHS-40 would be at the 5′ end of the next transgene, in the same orientation as is found in vivo. Five αP and 10 αPE transgenic lines were established. Transgene copy number was determined by Southern blot analysis and ranged from 1 to >100 (Table 1). Lines in which the transgene array contained deletions or rearrangements were excluded from the study.

Staining of whole embryos at 11.5 dpc with X-Gal revealed

TABLE 1. Expression of β-Gal in αP and αPE transgenic lines

Construct	Line	Copy no.	Expression	
			Erythroid[a]	Ectopic[b]
αP	αP-1	1	+	+
	αP-2	5	+	+
	αP-3	5–10	+	+
	αP-4	5–10	−	+
	αP-5	80	+	+
αPE	αPE-1	1–3	+	+
	αPE-2	2–5	+	+
	αPE-3	2–5	+	+
	αPE-4	10–15	+	+
	αPE-5	20	+	+
	αPE-6	20–30	+	+
	αPE-7	30–40	+	+
	αPE-8	50	+	−
	αPE-9	60	+	+
	αPE-10	>100	−	−

[a] Erythroid expression of the transgene was determined by the presence of blue-staining erythroid cells after staining of erythroid cell suspensions from 11.5-dpc mice in X-Gal for 24 h at 37°C. Up to 5×10^5 cells were examined for each construct. +, presence of transgene-expressing erythroid cells; −, absence of transgene-expressing erythroid cells.

[b] Ectopic expression of the transgene was determined by the presence of blue-staining nonerythroid tissues after staining of whole 11.5-dpc embryos in X-Gal for 5 h at 37°C. +, ectopic expression present after staining; −, ectopic transgene expression not discernible.

TABLE 2. Percentage of β-Gal-expressing cells, β-Gal activity per cell, and β-Gal activity per expressing cell in blood from 11.5-dpc embryos

Construct	Line	Copy no.	% of β-Gal-expressing cells at 11.5 dpc[a]	β-Gal activity per:	
				10^{10} cells at 11.5 dpc[b]	10^{10} expressing cells at 11.5 dpc[c]
αP	αP-1	1	<0.001	ND[d]	ND
	αP-2	5	1.0 (0.7)	6.5 (1.4)	650 (142)
	αP-3	5–10	<0.001	ND	ND
	αP-4	5–10	0	ND	ND
	αP-5	80	<0.001	ND	ND
αPE	αPE-1	1–3	97.5 (2.5)	480 (57)	492 (59)
	αPE-2	2–5	92.1 (8.2)	365 (47)	395 (51)
	αPE-3	2–5	21.9 (3.8)	62.0 (3.9)	284 (19)
	αPE-4	10–15	97.5 (2.5)	313 (95)	321 (98)
	αPE-5	20	56.1 (6.4)	212 (29)	377 (52)
	αPE-6	20–30	28.5 (2.7)	135 (18)	474 (62)
	αPE-7	30–40	9.0 (3.1)	30.6 (8.4)	341 (94)
	αPE-8	50	34.5 (1.9)	111 (21)	321 (60)
	αPE-9	60	1.7 (0.7)	5.2 (0.75)	306 (44)

[a] Percentage of erythroid cells that stain blue after staining of erythroid cell suspensions from 11.5-dpc embryos with X-Gal for 24 h at 37°C. A minimum of 200 cells were counted from three or more embryos, and the percentage is expressed as the mean with the standard deviation following in parentheses.

[b] β-Gal activity is expressed as the ΔA_{413} per minute of reaction per 10^{10} erythroid cells. At least three separate lysate preparations were assayed, and β-Gal activity is given as the mean with the standard deviation following in parentheses.

[c] β-Gal activities per expressing cell were calculated by correcting the activity obtained as described in footnote *b* with the percentage of blue-staining cells determined as described in footnote *a* and is expressed as the mean with the standard deviation following in parentheses.

[d] ND, not determined because the number of staining cells was so few that β-Gal activity was not detectable over background levels.

that the majority of lines had detectable levels of β-Gal in the erythroid tissues (Table 1). In addition to erythroid expression, most lines showed ectopic expression of the transgene at 11.5 dpc in patterns that vary from line to line. The patterns of ectopic expression differ markedly from line to line (data not shown), suggesting that the site of integration plays a major role. Lines made from both constructs show ectopic expression, which suggests that αHS-40 does not contain elements that suppress activity in nonerythroid lineages. We found that the ectopic expression of the transgene at 11.5 dpc appears to be pancellular (data not shown), in contrast to the heterocellular expression seen in erythroid cells (see below). It is notable that αHS-40 appears to have no effect on the pattern or extent of transgene expression except in erythroid cells (see below), consistent with its role as an erythroid cell-specific element.

The αPE-10 line failed to express β-Gal in erythroid cells. While Southern blot analysis did not show any major deletions of the transgene, it did reveal that this line contained a large number of copies of the transgene; its failure to express may be related to the silencing of large tandem arrays that has been described in *Drosophila melanogaster* (5, 6, 37).

The αHS-40 enhancer increases the proportion of erythroid cells that express the transgene. Expression of the transgene in erythroid cells of the lines was assessed both by X-Gal staining of erythroid cell suspensions and by β-Gal assays with lysates of erythrocytes (Table 2). As with other globin promoter-*lacZ* constructs that contain αHS-40, expression in erythrocytes is heterocellular or variegated (33). Prolonged incubation with the substrate does not increase the numbers of stained cells (33), the extremely sensitive fluorescence-activated cell sorter-Gal assay produces results that agree with those of X-Gal staining (34), and stimulation of erythropoiesis by phenylhydrazine treatment does not increase the proportion of staining cells (45a); for these reasons, we believe that X-Gal staining accurately detects all β-Gal-expressing cells. Table 2 shows the percentage of β-Gal-expressing erythroid cells in blood from each of the lines at 11.5 dpc. The percentage of expressing cells in each line does not bear a direct relationship to the number of transgene copies in the line; some high-copy-number lines show a low percentage of staining cells and vice versa, suggesting that the site of integration rather than copy number is the primary determinant of the extent of variegation.

The αP lines have extremely low numbers of expressing cells; in one of the five lines, no expressing cells were detected, and the highest expression was in line αP-2 at 1.0% at 11.5 dpc. Examination of cells stained with X-Gal for β-Gal expression permits a direct assessment of the proportion of cells expressing the transgene and detection of rare (one cell in a million) expressing cells. Previous studies have shown that in transgenic mice carrying the human α-globin gene without αHS-40, expression is either undetectable or extremely low (12, 26, 36, 40). In all of these studies, expression was determined at the RNA level with lysates of blood cells; low or undetectable levels could be due either to low levels in all erythroid cells or to a very low percentage of cells in which the transgene is active at high levels. The expressing cells in the αP mice, however, appear to stain as strongly as cells from the αPE lines (shown in Fig. 2 and below). Our result suggests that the low levels of expression in the earlier studies were produced by very small numbers of expressing cells.

Lines carrying the αPE construct express in markedly higher percentages of erythrocytes than do the αP lines. The percentage varies from 98% (αPE-1 and αPE-4) of erythroid cells expressing the transgene down to only 2% (αPE-9) at 11.5 dpc. While in both sets of mice there is variation from line to line in the percentage of expressing cells, the presence of the αHS-40 enhancer clearly increases the proportion of expressing cells by approximately 2 orders of magnitude.

Effect of the αHS-40 enhancer on β-Gal levels in expressing cells. We find that when heterocellularity is taken into account, all of the lines express the transgene at approximately the same level (Table 2); in all but one of the αP lines, the rarity of expressing cells did not permit direct quantitation, but expressing cells from these lines were seen to stain as strongly as those in other αP and αPE lines (Fig. 2). To obtain a measure of transgene activity in expressing cells, we assayed 11.5-dpc erythrocyte lysates for β-Gal activity and adjusted the levels obtained with the percentage of expressing cells determined by

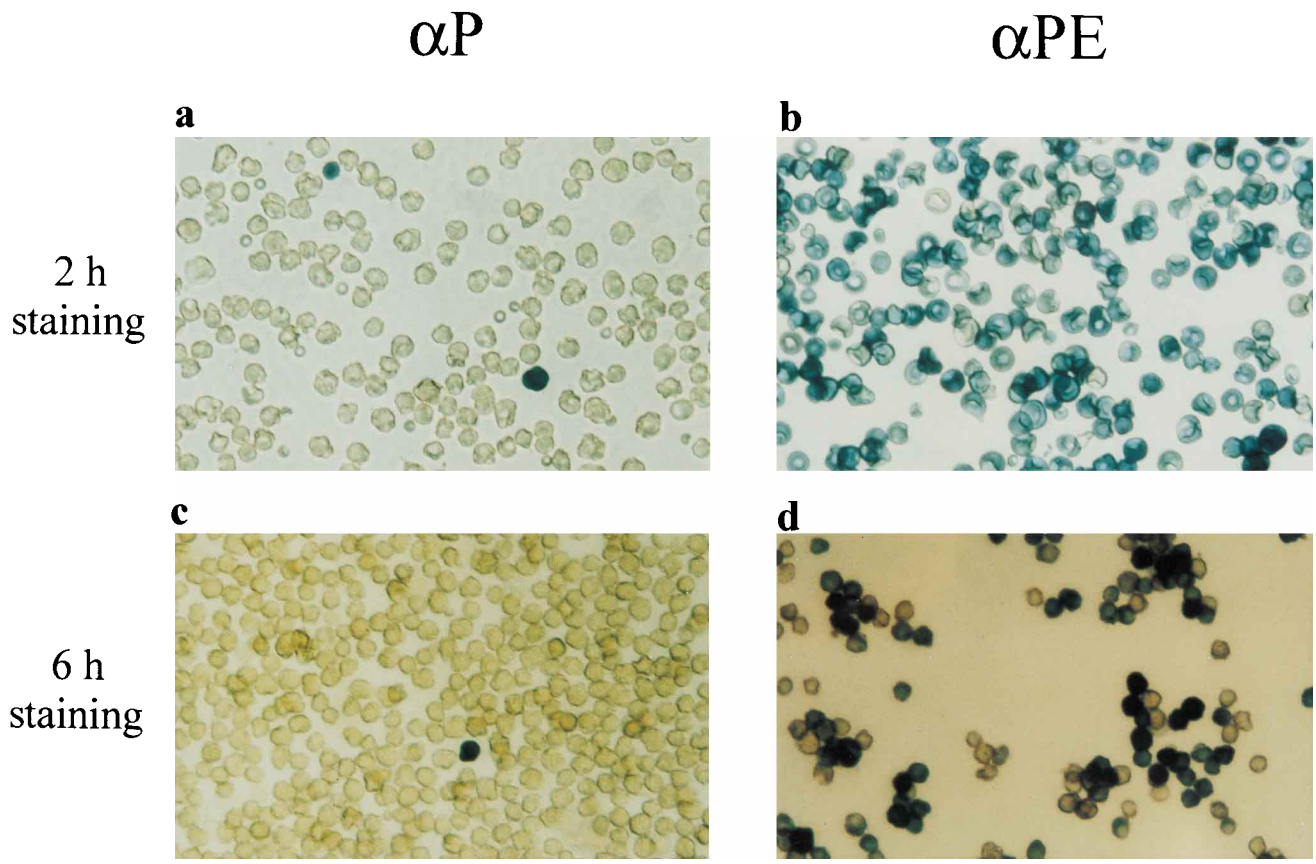

FIG. 2. X-Gal staining of 11.5-dpc erythrocytes from αP and αPE transgenic mice. Erythroid cell suspensions from αP and αPE 11.5-dpc embryos were fixed, washed, and stained with X-Gal simultaneously under identical conditions. The cells were photographed after timed incubations of 2 h (a and b) or 6 h (c and d) at 37°C. After 2 h of incubation with X-Gal, the line αP-2 (a), in which 1% of cells express β-Gal, shows some β-Gal-expressing erythroid cells that stain at least as intensely blue those from the line αPE-1 (b), in which 98% of cells express β-Gal. After 6 h of incubation, the line αP-1 (c), in which <0.001% of cells express β-Gal, shows an expressing erythroid cell that stains as intensely blue as those from line αPE-5 (d), in which 56% of cells express β-Gal.

staining with X-Gal (Table 2). This provides a value for the β-Gal activity per expressing cell, which is similar in all of the αPE lines regardless of copy number. This is consistent with other studies that show αHS-40 does not confer copy number dependence (16, 39, 40). Transgenic mice with a β-locus control region (LCR)–β-globin promoter–*lacZ* construct express at least 10 times more β-Gal per expressing cell than do the αPE mice, so that the level of β-Gal is not likely to have reached a limiting level in these mice (52a).

Only rare cells express β-Gal in most of the αP lines (αP-1, αP-3, and αP-4), so the activity in blood lysates of these lines was undetectable, and a numerical value for expression per cell could not be derived. Only αP-2 expresses β-Gal in a sufficient number of erythrocytes to permit measurement of the expression per cell. The calculated β-Gal activity per expressing cell in this line approximates that seen in the αPE lines (Table 2). Figure 2 further demonstrates that the intensity of X-Gal staining of β-Gal-positive cells in αP lines (αP-1 and αP-2) is comparable to that in αPE lines (αPE-1 and αPE-5) after simultaneous, timed incubation with the substrate. Similar experiments have been performed with all of the αP lines, and in every case, the intensity of staining is similar to that seen in the αPE lines (data not shown). Thus, the effect of αHS-40 on expression of the *lacZ* transgene is to increase the proportion of cells in which the transgene is expressed, without any apparent effect on the level of expression in those cells.

The α-globin promoter in β-Gal-negative cells from expressing lines is not heavily methylated. We investigated the methylation status of the α-globin promoter in β-Gal-negative cells to determine if it differs from that in β-Gal-expressing cells. DNA was extracted from 12.5-dpc erythroid cells and digested with the methylation-sensitive restriction endonucleases *Eag*I and *Sma*I, for which there are several sites in the α-globin promoter (Fig. 3A). Digestion of genomic DNA with *Pst*I and *Eco*RV will generate a 1.7-kb fragment which contains both the α-globin promoter and the 5′ portion of the *lacZ* gene. Additional digestion with *Eag*I or *Sma*I will yield smaller fragments, only if the DNA is unmethylated at their recognition sites. Figure 3B shows that the promoter is not methylated at these sites in either αP- or αPE-expressing lines, even though in some cases, most of the erythroid cells are not expressing the transgene: αP-2 has 1% β-Gal-positive cells, and αPE-3 has 20% β-Gal-positive cells. Therefore, methylation does not correlate with the extent of silencing (variegation) observed in expressing transgenic lines. Only αPE-10, a high-copy line which completely fails to express β-Gal, shows methylation of the transgene at these sites in the promoter. In the case of αPE-10, we do not know if methylation is the primary cause of the lack of expression from the transgene or if it is a secondary effect of inactivation by other mechanisms. It is clear from these results that the methylation status of the globin promoter in β-Gal-negative cells from expressing lines

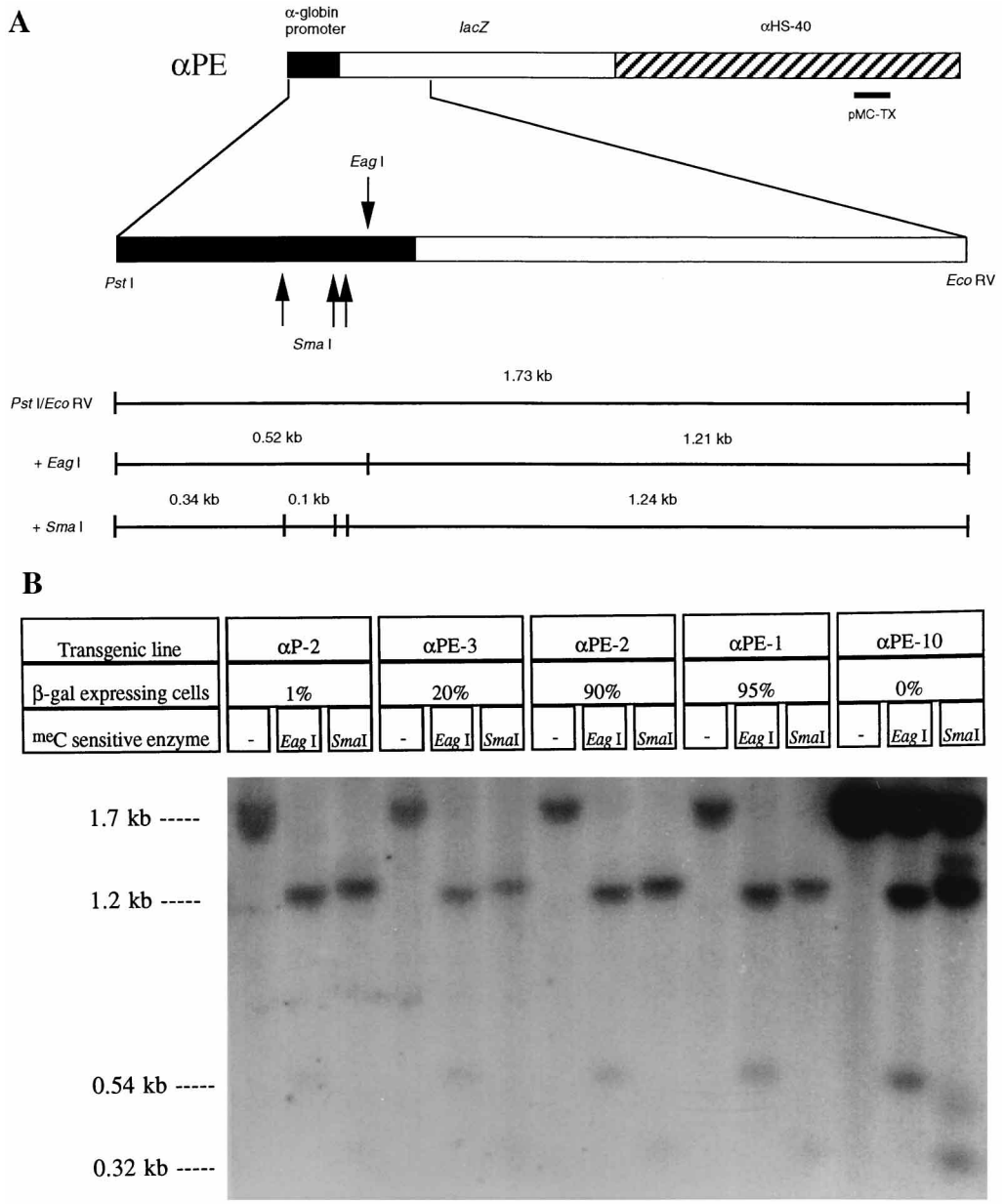

FIG. 3. Methylation status of αP and αPE transgenes in lines with various levels of expression. (A) Diagram of the αPE transgene showing the 1.7-kb *Pst*I-*Eco*RV fragment encompassing the α-globin promoter and 5′ portion of the *lacZ* gene. Bars below show relative positioning along the 1.7-kb fragment of the methylation (^{me}C)-sensitive restriction enzymes *Eag*I and *Sma*I and the fragment sizes expected if the sites are unmethylated. (B) Southern blot analysis of genomic DNA isolated from 12.5-dpc embryonic erythrocytes. DNA from various lines was digested with *Pst*I and *Eco*RV only or with *Pst*I and *Eco*RV and either *Eag*I or *Sma*I as indicated, and approximate fragment sizes are shown on the left. αP-2, αPE-3, αPE-2, and αPE-1 all show complete digestion of the 1.7-kb fragment indicating lack of methylation of the transgene at these sites in the promoter, even though in some cases the proportion of cells expressing β-Gal is small. The 1.7-kb fragment remains largely intact in the case of the nonexpressing line, αPE-10, showing that this transgene is heavily methylated.

is not the same as that found in cells from completely inactive lines.

Expression of the transgene declines during development. Although in humans the α-globin gene is expressed in the embryo along with ζ-globin, it is the major adult α-like gene and consequently remains active throughout adult life. We found, however, that mice carrying the αPE construct exhibit a decline in β-Gal activity in erythroid cell lysates as development proceeds (Fig. 4A); this decline continues well past the point at which definitive erythropoiesis is established. When the percentage of β-Gal-expressing erythroid cells was determined throughout this period (Fig. 4B), it exhibited a decline that parallels that of the total β-Gal activity in blood lysates. The percentage of β-Gal-expressing cells decreased at different rates in each of the lines. Some had decreased below 1% by 2 months after birth (αPE-8), while most were between 1 and 10% at this time point. Of these, some stabilized and continued to express at that percentage past 1 year of age (αPE-3), while others continued to decrease as the age increased (αPE-1, αPE-4, and αPE-5). In still other lines, 20 to 40% of erythroid cells expressed β-Gal at 2 months or more, but the percentage gradually decreased with age also (data not shown).

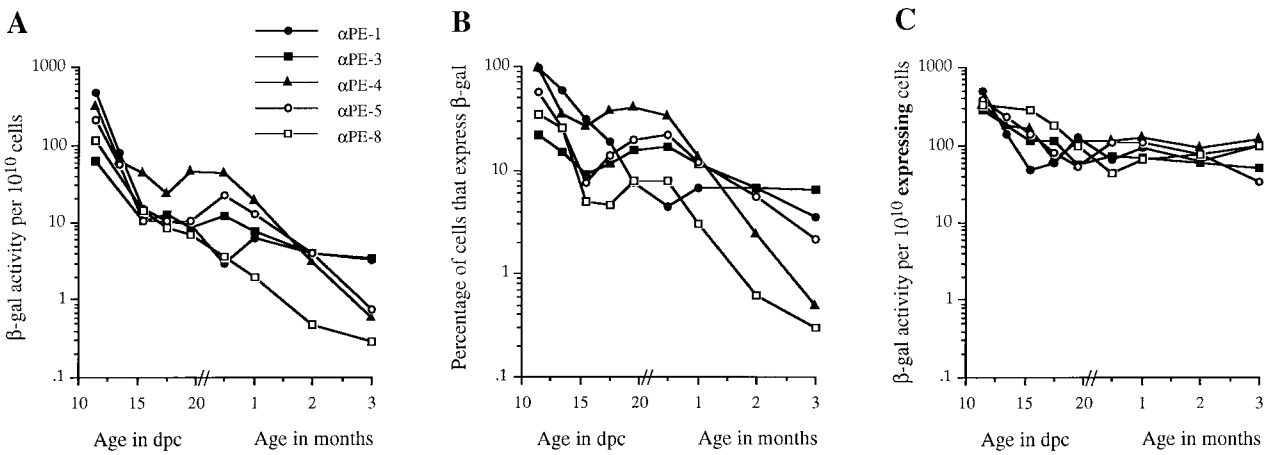

FIG. 4. Transgene activity in erythroid cells through development of the transgenic lines αPE-1, αPE-3, αPE-4, αPE-5 and αPE-8. (A) β-Gal activity per 10^{10} erythroid cells with increasing age of the mouse. β-Gal activity is defined as ΔA_{413} per minute of reaction. Each experimental point represents a mean of three or more individuals. Error bars have been omitted for clarity, but generally the standard deviation was within 25% of the mean. (B) Percentage of cells that express β-Gal with increasing age of the mouse. Each experimental point represents a mean of three or more individuals. Error bars have been omitted for clarity, but the standard deviation was within 20% of the mean. (C) β-Gal activity per 10^{10} expressing cells. Values were calculated by correcting the values obtained in panel A with the mean percentage of cells that express β-Gal shown in panel B. Each experimental point represents a mean of three or more individuals.

When β-Gal activity per expressing cell was calculated at a number of time points throughout development (by correcting β-Gal activity per cell for the percentage of expressing cells), most lines still showed a decrease, but it was greatly reduced (Fig. 4C). Thus, while there was a 20- to >500-fold decrease in total transgene expression between 11.5 dpc and 3 months after birth, the β-Gal activity per expressing cell dropped only by approximately 5-fold during this period. Clearly the major component of the age-dependent decrease in β-Gal expression in erythroid cells is the decrease in the proportion of expressing cells. The small decline in the level of expression per active cell may correspond to the switch in cell type from primitive to definitive erythrocytes which occurs at this point (44).

DISCUSSION

We have observed that addition of a globin enhancer to a transgene has the effect of increasing the proportion of erythrocytes that express the transgene, without any measurable effect on transgene expression level in expressing cells. While this effect is contrary to the prevailing model of enhancer action, it is consistent with several experiments that have described the enhancer effect as one of increasing the establishment and maintenance of expression, rather than transcription rate. Silencing of the transgene is not associated with increased promoter methylation, except in lines that completely fail to express the transgene. Silencing trends to increase with age of individual mice, but the pattern of expression during ontogeny does not change in successive generations. The silencing we observed is not classic position-effect variegation (PEV); it occurs in every transgenic line, making it extremely unlikely that in all cases it is due to integration near centromeric heterochromatin. The element we have studied, αHS-40, is an enhancer that has been found not to act as an LCR (16, 39, 40). Our findings are thus consistent with a model in which an enhancer acts to increase the likelihood that transcription will be established, maintained, or both during differentiation.

Variegation of transgene expression has been widely observed, and we speculate that analysis of expression on a single-cell level would identify many more instances (21). Variegation appears to be similar to a number of silencing phenomena which have in common the placement of an active gene within or near repressive chromatin (14, 28, 46). PEV was described in chromosomal translocations that place a marker gene near constitutive heterochromatin, resulting in stochastic and clonally heritable inactivation of the marker. A recent report found that a human CD2 transgene is subject to PEV and that this PEV is suppressed by inclusion of an LCR in the transgene (10); this LCR element did not act as an enhancer in other assays. LCRs direct high-level, tissue-specific expression of linked genes in transgenic mice, and the expression is independent of the site of integration in the host genome (11, 27, 31, 38). The results presented here suggest that suppression of variegation can also be achieved by the addition of enhancer elements to a transgene construct. Since enhancers are found adjacent to many eukaryotic genes, while only a handful of LCRs have been characterized, this finding is of general significance.

The αHS-40 element acts as a transcriptional enhancer in both transient and stable expression assays in cell culture (29, 32, 41) and when included in constructs in transgenic mice (16, 39, 40). αHS-40 is classified as an enhancer rather than an LCR because it does not confer position independence and copy number dependence in the transgenic assays. It is bound by a group of nuclear factors that also bind and activate other enhancers and promoters of erythroid cell-specific genes (17, 45), and naturally occurring deletions of αHS-40 from the α-globin locus in humans result in a severe down-regulation of α-globin expression (13, 20, 35). However, in all of the experiments cited above, the effect of the αHS-40 was not assayed on a cell-by-cell basis. We find that the αHS-40 enhancer strongly suppresses variegation of a *lacZ* transgene in erythroid cells. Within expressing cells, its presence does not appear to significantly affect the level of transgene expression, although this judgment must be tempered by the fact that the rarity of expressing cells in most promoter-only lines permits only a crude assessment of expression level.

This work represents the first cell-by-cell assay of enhancer activity performed in a transgenic animal. It adds to the growing body of evidence, derived from experiments with cultured cells, supporting the view that enhancers act primarily to increase the probability of a gene establishing a transcriptionally

active state (23, 24, 49, 50, 52). Suppression of silencing by transcriptional activators has also been described in *Drosophila* and yeast species (1, 9). Our results suggest that the presence of a transcriptional enhancer in the transgenic construct serves to establish and maintain a transcriptionally active region by antagonizing formation of a stable repressed state, in effect insulating a transcription unit from flanking chromatin. Since many enhancers are bound by tissue-specific factors, they may permit a gene to remain in an active state only in appropriate cell types.

It is striking that in every mouse line carrying a globin transgene we have analyzed (in this and other studies the total is greater than 40), expression in erythrocytes was variegated at some developmental stage, while in studies of transgenes expressed in other lineages, only a small proportion of lines exhibited variegation (7, 21). We suggest that this characteristic of transgenes expressed in erythroid cells is attributable to the very heterochromatic nature of the erythroid nucleus, where virtually any integration site will be subjected to a strongly repressive influence. During terminal erythroid differentiation, the nucleus is progressively condensed and eventually becomes transcriptionally inactive. The globin loci, which are transcribed during the terminal phase of erythroid differentiation when heterochromatinization is occurring, are adapted to remain active in this context (2). However, most sites into which a transgene may randomly integrate will be inactivated during terminal differentiation. An enhancer linked to the promoter may function to prevent the formation of stable repressive structures that silence the construct, increasing the likelihood of the transgene maintaining an active state during erythroid differentiation. Clearly the α-globin promoter, even in the presence of αHS-40, is not able to completely suppress variegation in 100% of erythroid cells, as must occur when it lies in its normal chromosomal context. We may be missing important *cis*-acting regulatory elements, perhaps contained in the α-globin coding sequence or surrounding regions, which have not been included in the constructs. Alternatively, the chromatin in which the α-globin cluster usually resides may not be as repressive as that found in much of the erythroid nucleus. In fact, Vyas et al. (48) have shown that the α-globin cluster lies in a region of chromatin structure that is open in all cell types.

Gene inactivation is often found to correlate with methylation (4), although it remains unclear whether methylation is the cause or consequence of the inactivation. We have found high levels of transgene methylation in lines in which the transgene is completely inactive in all tissues and at all stages of development. However, in lines containing both β-Gal-positive and -negative cells, the transgene appears to be unmethylated in all cells. Although methylation could be occurring at some specific site which is not detected in this assay, these experiments suggest that methylation is unlikely to be responsible for the inactivity of the transgene in variegating lines. We speculate that methylation of the transgene may be associated with inactivity only when this is a germline event, rather than the stochastic inactivation characteristic of most lines.

We noted a strong tendency for the extent of variegation to increase with age in these mice; the same effect has been observed in mice carrying several other constructs (34) and may be responsible for the variable down-regulation of expression observed by Sharpe et al. (40) with α-globin constructs containing no prokaryotic gene sequence. Our observations are similar to findings by others that foreign genes are progressively silenced upon prolonged incubation in tissue culture (50); they confirm that this silencing occurs in whole animals and is not merely the aberrant behavior of transgenes in transformed cells maintained under artificial conditions. At present, the cause of the decrease in expressing cells is not understood. While it is not suggested that genes present in their normal chromosomal locations undergo such silencing, it is clear that such a decline is likely to be a confounding factor in studies of developmental regulation of genes in transgenic mice and in situations in vivo in which continued expression of the transgene is required.

The findings of this study may well be generally applicable to the action of enhancers in transgenic systems; we predict that when addition of an enhancer to a transgene increases expression, it will do so by increasing the proportion of cells expressing the transgene (suppressing variegation). Taken together with other work, this study suggests that enhancers may act primarily or entirely by increasing the ability of a gene to maintain activity; we speculate that the presence of a sufficient concentration of such elements may be necessary to provide the virtual assurance of activity in the appropriate lineage that is characteristic of tissue-specific loci such as the globin loci. Transgenic mice will provide an excellent method of studying this aspect of enhancer action.

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REFERENCES

1. **Aparicio, O. M., and D. E. Gottschling.** 1994. Overcoming telomeric silencing a trans-activator competes to establish gene expression in a cell cycle-dependent way. Genes Dev. **8:**1133–1146.
2. **Bonanou-Tzedaki, S. A., and H. R. V. Arnstein.** 1987. Macromolecular synthesis and degradation during terminal erythroid cell development. Biomed. Biochim. Acta **46:**121–149.
3. **Bradl, M., L. Larue, and B. Mintz.** 1991. Clonal coat variation due to a transforming gene expressed in melanocytes of transgenic mice. Proc. Natl. Acad. Sci. USA **88:**6447–6451.
4. **Cedar, H.** 1988. DNA methylation and gene activity. Cell **53:**3–4.
5. **Dorer, D.** Do transgene arrays form heterochromatin in vertebrates? Transgene Res., in press.
6. **Dorer, D. R., and S. Henikoff.** 1994. Expansions of transgene repeat cause heterochromatin formation and gene silencing in Drosophila. Cell **77:**993–1002.
7. **Elliot, J. I., R. Festenstein, M. Tolaini, and D. Kioussis.** 1995. Random activation of a transgene under the control of a hybrid hCD2 locus control region/Ig enhancer regulatory element. EMBO J. **14:**575–584.
8. **Enver, T., A. J. Ebens, W. C. Forrester, and G. Stamatoyannopoulos.** 1989. The human β-globin locus activation region alters the development fate of a human fetal globin gene in transgenic mice. Proc. Natl. Acad. Sci. USA **86:**7033–7037.
9. **Farkas, G., J. Gausz, M. Galloni, G. Reuter, H. Gyurkovics, and F. Karch.** 1994. The trithorax-like gene encodes the Drosophila GAGA factor. Nature **371:**806–808.
10. **Festenstein, R., M. Tolaini, P. Corbella, C. Mamalaki, J. Parrington, M. Fox, A. Miliou, M. Jones, and D. Kioussis.** 1996. Locus control region function and heterochromatin induced position effect variegation in transgenic mice. Science **271:**1123–1126.
11. **Grosveld, F., G. Blom van Assendelft, D. R. Greaves, and G. Kollias.** 1987. Position-independent, high-level expression of the human β-globin gene in transgenic mice. Cell **51:**975–985.
12. **Hanscombe, O., M. Vidal, J. Kaeda, L. Luzzatto, D. R. Greaves, and F. Grosveld.** 1989. High-level, erythroid-specific expression of the human α-globin gene in transgenic mice and the production of human hemoglobin in murine erythrocytes. Genes Dev. **3:**1572–1581.
13. **Hatton, C., A. O. M. Wilkie, H. C. Drysdale, W. G. Wood, M. A. Vickers, J. Sharpe, H. Ayyub, I.-M. Pretorius, V. J. Buckle, and D. R. Higgs.** 1990. Alpha thalassemia caused by a large (62 kb) deletion upstream of the human α-globin gene cluster. Blood **76:**221–227.
14. **Henikoff, S.** 1992. Position effect and related phenomena. Curr. Opin. Genet. Dev. **2:**907–912.

15. **Herbomel, P., B. Bourachot, and M. Yaniv.** 1984. Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell **39:**653–662.
16. **Higgs, D. R., W. G. Wood, A. P. Jarman, J. Sharpe, J. Lida, I.-M. Pretorius, and H. Ayyub.** 1990. A major positive regulatory region located far upstream of the human α-globin gene locus. Genes Dev. **4:**1588–1601.
17. **Jarman, A. P., W. G. Wood, J. A. Sharpe, G. Gourdon, H. Ayyub, and D. R. Higgs.** 1991. Characterization of the major regulatory element upstream of the human α-globin gene cluster. Mol. Cell. Biol. **11:**4679–4689.
18. **Katsuki, M., M. Sato, M. Kimura, M. Yokoyama, K. Kobayashi, and T. Nomura.** 1988. Conversion of normal behavior to shiverer by myelin basic protein antisense cDNA in transgenic mice. Science **241:**593–595.
19. **Lewin, B.** 1990. Genes IV. Oxford University Press, New York, N.Y.
20. **Liebhaber, S. A., E.-U. Griese, I. Weiss, F. E. Cash, H. Ayyub, D. R. Higgs, and J. Horst.** 1990. Inactivation of human α-globin gene expression by a de novo deletion located upstream of the α-globin gene cluster. Proc. Natl. Acad. Sci. USA **87:**9431–9435.
21. **Martin, D. I. K., and E. Whitelaw.** 1996. The vagaries of variegating transgenes. BioEssays **18:**919–923.
22. **Mintz, B., and M. Bradl.** 1991. Mosaic expression of a tyrosinase fusion gene in albino mice yields a heritable striped coat color pattern in transgene homozygotes. Proc. Natl. Acad. Sci. USA **88:**9643–9647.
23. **Moon, A. M., and T. J. Ley.** 1990. Conservation of the primary structure, organization, and function of the human and mouse β-globin locus-activation regions. Proc. Natl. Acad. Sci. USA **87:**7693–7697.
24. **Moreau, P., R. Hen, B. Wasylyk, R. Everett, M. P. Gaub, and P. Chambon.** 1981. The SV40 72 base pair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants. Nucleic Acids Res. **9:**6047–6068.
25. **Morley, B. J., C. A. Abbott, J. A. Sharpe, J. Lida, P. S. Chan-Thomas, and W. G. Wood.** 1992. A single β-globin locus control region element (5′ hypersensitive site 2) is sufficient for developmental regulation of human globin genes in transgenic mice. Mol. Cell. Biol. **12:**2057–2066.
26. **Palmiter, R. D., and R. L. Brinster.** 1986. Germ-line transformation of mice. Annu. Rev. Genet. **20:**465–499.
27. **Palmiter, R. D., E. P. Sandgren, D. M. Koeller, and R. L. Brinster.** 1993. Distal regulatory elements from the mouse metallothionein locus stimulate gene expression in transgenic mice. Mol. Cell. Biol. **13:**5266–5275.
28. **Pirrotta, V., and L. Rastelli.** 1994. White gene expression, repressive chromatin domains and homeotic regulation in Drosophila. BioEssays **16:**549–556.
29. **Pondel, M., M. George, and N. J. Proudfoot.** 1992. The LCR-like α-globin positive regulatory element functions as an enhancer in transiently transfected cells during erythroid differentiation. Nucleic Acids Res. **20:**237–243.
30. **Pravtcheva, D. D., T. L. Wise, N. J. Ensor, and F. J. Ruddle.** 1994. Mosaic expression of an HPRT transgene integrated in a region of Y heterochromatin. J. Exp. Zool. **268:**452–468.
31. **Reitman, M., E. Lee, H. Westphal, and G. Felsenfeld.** 1990. A enhancer/locus control region is not sufficient to open chromatin. Nature **348:**749–752.
32. **Ren, S., X.-N. Luo, and G. F. Atweh.** 1993. The major regulatory element upstream of the α-globin gene has classical enhancer and inducible enhancer activity. Blood **4:**1058–1066.
33. **Robertson, G., D. Garrick, W. Wu, M. Kearns, D. Martin, and E. Whitelaw.** 1995. Position-dependent variegation of globin transgene expression in mice. Proc. Natl. Acad. Sci. USA **92:**5371–5375.
34. **Robertson, G., D. Garrick, M. Wilson, D. Martin, and E. Whitelaw.** 1996. Age-dependent silencing of globin transgene expression in the mouse. Nucleic Acids Res. **24:**1465–1471.
35. **Romao, L., L. Osorio-Almeida, D. R. Higgs, J. Lavinha, and S. A. Leibhaber.** 1991. α-Thalassemia resulting from deletion of regulatory sequences far upstream of the α-globin structural genes. Blood **78:**1589–1595.
36. **Ryan, T. M., R. R. Behringer, T. M. Townes, R. D. Palmiter, and R. L. Brinster.** 1989. High-level erythroid expression of human α-globin genes in transgenic mice. Proc. Natl. Acad. Sci. USA **86:**37–41.
37. **Sabl, J. F., and S. Henikoff.** 1996. Copy number and orientation determine the susceptibility of a gene to silencing by nearby heterochromatin in Drosophila. Genetics **142:**447–458.
38. **Schedl, A., L. Montoliu, G. Kelsey, and G. Schutz.** 1993. A yeast artificial chromosome covering the tyrosinase gene confers copy number-dependent expression in transgenic mice. Nature **362:**258–261.
39. **Sharpe, J. A., P. S. Chan-Thomas, J. Lida, H. Ayyub, W. G. Wood, and D. R. Higgs.** 1992. Analysis of the human α globin upstream regulatory element (HS-40) in transgenic mice. EMBO J. **11:**4565–4572.
40. **Sharpe, J. A., D. J. Wells, E. Whitelaw, P. Vyas, D. R. Higgs, and W. G. Wood.** 1993. Analysis of the human α-globin gene cluster in transgenic mice. Proc. Natl. Acad. Sci. USA **90:**11262–11266.
41. **Sharpe, J. A., R. J. Summerhill, P. Vyas, G. Gourdon, D. R. Higgs, and W. G. Wood.** 1993. Role of upstream DNase I hypersensitive sites in the regulation of human α globin gene expression. Blood **82:**1666–1671.
42. **Singer, M., and P. Berg.** 1991. Genes and genomes. University Science Books, Mill Valley, Calif.
43. **Stamatoyannopoulos, G., B. Josephson, J.-W. Zhang, and Q. Li.** 1993. Developmental regulation of human γ-globin genes in transgenic mice. Mol. Cell. Biol. **13:**7636–7644.
44. **Stamatoyannopoulos, G., and A. Nienhuis.** 1994. Hemoglobin switching, p. 107–156. *In* G. Stamatoyannopoulos (ed.), The molecular basis of blood diseases. W. B. Saunders, Philadelphia, Pa.
45. **Strauss, E. C., N. C. Andrews, D. R. Higgs, and S. H. Orkin.** 1992. In vivo footprinting of the human α-globin locus upstream regulatory element by guanine and adenine ligation-mediated polymerase chain reaction. Mol. Cell. Biol. **12:**2135–2142.
45a. **Sutherland, H. G. E., and E. Whitelaw.** Unpublished observations.
46. **Tartof, K. D.** 1994. Position effect variegation in yeast. BioEssays **16:**713–714.
47. **Treisman, R., and T. Maniatis.** 1985. Simian virus 40 enhancer increases number of RNA polymerase II molecules on linked DNA. Nature **315:**72–75.
48. **Vyas, P., M. A. Vickers, D. L. Simmons, H. Ayyub, C. F. Craddock, and D. R. Higgs.** 1992. Cis-acting sequences regulating expression of the human α-globin cluster lie within constitutively open chromatin. Cell **69:**781–793.
49. **Walters, M. C., S. Fiering, J. Eidemiller, W. Magis, M. Groudine, and D. I. K. Martin.** 1995. Enhancers increase the probability but not the level of gene expression. Proc. Natl. Acad. Sci. USA **92:**7125–7129.
50. **Walters, M. C., W. Magis, S. Fiering, J. Eidemiller, D. Scalzo, M. Groudine, and D. I. K. Martin.** 1996. Transcriptional enhancers act in cis to suppress position-effect variegation. Genes Dev. **10:**185–195.
51. **Weber, F., and W. Schaffner.** 1985. Simian virus 40 enhancer increases RNA polymerase density within the linked gene. Nature **315:**75–77.
52. **Weintraub, H.** 1988. Formation of stable transcription complexes as assayed by analysis of individual templates. Proc. Natl. Acad. Sci. USA **85:**5819–5823.
52a. **Whitelaw, E.** Unpublished observations.