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Position effects in mice carrying a *lacZ* transgene in *cis* with the β-globin LCR can be explained by a graded model

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ABSTRACT

We studied transgenic mice carrying the *lacZ* reporter gene linked to the erythroid-specific β-globin promoter and β-globin locus control region (LCR). Previously, we had demonstrated that the total level of expression of β-galactosidase enzyme, which is the product of the *lacZ* gene, varies widely between different transgenic mice due to position effects at the sites of transgene integration. Here, using the X-gal based *in situ* assay for β-galactosidase activity, we found that the percent erythroid cells that expressed the transgene also varied widely between the mice. Moreover, a kinetic analysis showed that the average β-galactosidase content per expressing cell varied both between samples of different transgenic descent and between erythroid cells within each sample, demonstrating that the variable expression of this *lacZ* transgene was being controlled in a graded manner. These results suggest that the β-globin LCR enhancers function through a graded model, which is described, rather than the binary mechanism that has been proposed previously for other enhancers.

INTRODUCTION

When transgenes integrate into the genome of mice or other organisms, they are generally prone to a phenomenon called position effects or position-dependent expression (reviewed in 1,2). The levels of expression show a wide variation between different sites of integration in the genome with no relationship to transgene copy number. These position effects are thought to be the result of the influence of neighboring chromatin structure on the functioning of enhancers controlling expression of the transgene. Thus, by studying position-effects we can gain insights into the mechanism of up-regulation of gene transcription by enhancers.

Originally, it was proposed that enhancers increase the transcription initiation rate of a gene by increasing the density of RNA polymerase molecules transcribing it (3,4). However, several studies have disputed this rate model. For example, using an assay for *lacZ* transgene activity that detects expression in individual cells, Walters *et al*. (5) found that enhancers increase the proportion of expressing cells, but not the level of expression in individual cells. To explain this result, they proposed the binary model, which suggests that the level of expression is always the same in each transcribing cell, but only some cells are able to form an active transcription complex. By affecting this binary (on/off) switch, enhancers increase the percentage of cells that form an active transcription complex. A similar hypothesis was originally put forth by Weintraub (6) and the possibility was also discussed earlier by Moreau *et al*. (7). More recently, Walters *et al*. showed that enhancers prevented transgene repression that occurred upon removal of drug selection from stably transfected cell clones (8). They therefore suggested that enhancers increase the number of cells able to undergo active transcription by preventing gene repression in individual cells.

It is well known that integration of a transgene near telomeric or centromeric regions in yeast or *Drosophila* (reviewed in 9,10), as well as in mice (reviewed in 11), can result in heterocellular expression of a transgene such that it is expressed in some cells but not in others. This phenomenon is called position effect variegation. In looking at expression in individual red blood cells of transgenic mice carrying a *lacZ* reporter gene under the control of α-globin regulatory sequences, Robertson *et al*. (12) found that position effects resulted in a variable number of expressing cells between different transgenic mouse lines, but did not appear to greatly affect the level of expression per cell. Moreover, the addition of an enhancer was found to increase the number of expressing cells with no apparent effect on the level of transcription in individual cells (13). These observations support the notion that position effects and enhancer function can be controlled by a binary model in some cases. However, the binary model alone may not fit all situations. For example, position effects on expression of the *white* gene in *Drosophila* sometimes result in variegation that can be seen as a mosaic (or variegated) eye color pattern. This is caused by the fact that some cells do not express the transgene at all while all other cells express at wild-type levels, as the binary model would predict. In contrast, many position effects on the *white* gene result in a variety of eye colors, ranging from light yellow, to orange, brown and dark red, without apparent cell to cell variegation (14). Similarly, even when an *ade6* transgene is integrated near a centromeric region in yeast, cells of a variable color between white and red arise, suggesting that there can be a gradient of transgene expression

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between individual cells (15). Thus, an alternative mechanism to the binary model may function in some cases.

Locus control regions (LCRs) are DNA elements that can overcome position effects (reviewed in 16). LCRs have been isolated from several gene loci and they are often associated with enhancers. However, the ability to enhance gene expression and the ability to confer position-independent expression onto transgenes appear to be independent and separable functions of LCRs.

To date, the best characterized LCR is that of the human β-globin gene locus (reviewed in 17,18). The β-globin locus contains five active genes (ε, Gγ, Aγ, δ and β) that are arranged in their developmental order of expression and all five genes rely on an LCR for high levels of expression. This β-globin LCR is located ~15 and 60 kb upstream of the ε- and β-globin genes, respectively, and it is signified by five regions of DNAse I hypersensitive sites (HS). Three of these HS regions have enhancer activities that can act independently of one another to give partial levels of expression (19 and references therein). How the full β-globin LCR protects against position effects is unresolved. It may require at least three of the HS regions (20), and may also involve specific sequences flanking the globin genes (21–23).

If the enhancers of the LCR act via the binary mechanism as proposed (5), one might predict the LCRs position-independence function is to prevent repression in all cells so that they all express at the same level. However, it has been shown that the genes of the β-globin locus compete for activation by the LCR during development (24–26), such that when a γ- and β-globin gene are together with the LCR in transgenic mice, the γ-globin gene is only expressed during embryonic and early fetal life, while the β-globin gene is expressed only in late fetal and adult life. This is so, even though the embryonic cells are perfectly competent to express the β-globin gene, since if the β-globin gene is alone with the LCR it is expressed at all stages of development in mice (24–26). Moreover, Wijgerde *et al.* showed that during the switching process the cells flip-flop between transcribing the γ- and β-globin gene from the same chromosome (27). In addition, some transgenic mice carrying a position-dependent γ-globin-LCR construct gave a heterogeneous cell to cell intensity of staining with γ-globin antibodies (28,29), and erythroid colonies isolated from patients with heterocellular hereditary persistence of fetal hemoglobin, a human disorder in which there is an increase in γ-globin producing cells in adults, show a heterogeneity in the amount of γ-globin production (30). Taken together, these results show that activation of the LCR enhancers does not necessarily mean that all cells will express a given gene at the same level. Therefore, although the LCR action may involve a binary mechanism, this model alone fails to explain all observations.

To understand how the β-globin LCR brings about position-independent expression, we need to know how the LCR enhancers will act on a globin gene promoter under a situation where the ability of the LCR to protect against position effects has been crippled. Recently, we showed that a *lacZ* transgene linked to the β-globin gene promoter in *cis* with the LCR is highly susceptible to position effects in mice (21). That this same transgene gave full levels of expression in individual drug-selected, transfected cell culture clones suggested that the LCR enhancers were functioning, but the LCRs' ability to prevent position effects does not work with such a transgene in mice. Here we look at *lacZ* transgene expression in individual blood cells of >40 different transgenic mice. We show that expression on a cell to cell basis is graded both within a single transgenic sample and between different samples. Thus, the β-globin LCR enhancers function through a graded mechanism that is described.

MATERIALS AND METHODS

Transgenic mice

The generation of transgenic mice used in this study has been described (21). Briefly, the βA transgene contains the *lacZ* coding sequences, followed by Simian Virus 40 (SV40) polyadenylation signals, linked to a minimal β-globin promoter (–87 to +32 relative to the Cap site) in *cis* with the LCR from the β-globin μ-locus plasmid (31). In the βB, βC, βD and βE transgenes the β-globin promoter extends from –800 to +32. βB is otherwise identical to βA. In βC and βD, sequences starting from the *Bam*HI site in the second intron to 1.7 kb downstream of the polyadenylation signal of the β-globin gene were cloned 3′ to the *lacZ* sequences in βB. For βC the SV40 polyadenylation signals were left intact so that the further β-globin gene sequences are not transcribed, while the SV40 polyadenylation signals were removed in the case of the βD construct. The βE transgene contains the whole β-globin gene, from –800 to 1.7 kb past the polyadenylation signal, with the *lacZ* sequences (without the SV40 polyadenylation signals) cloned directly into the *Nco*I site situated at the β-globin protein initiation codon. As indicated in Table 1, transgenic animals were either studied as founders isolated 15 days after microinjection or stable lines were produced and 15.5 day old fetuses of third and further generations were studied.

β-galactosidase assays

For *in situ* assays with the X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) substrate, one quarter of a fetal liver was dispersed into 100 μl of 0.25 M Tris–HCl (pH 8.0). Ten μl of the cell suspension was deposited into a well of a 96 well tissue culture plate, and then the cells were fixed by incubating in 100 μl of phosphate buffered saline (PBS), containing 0.2% glutaraldehyde, for 10 min. The cells were rinsed with PBS and incubated in the dark at 37°C in PBS containing 0.1 M X-gal, 0.01 M potassium ferrocyanide, 0.01 M potassium ferricyanide and 1 mM $MgCl_2$, for either 24 h, after which time no further staining was observed, or for the times indicated in the figures. Measurements of the percent blue stained cells and photographs were done under phase contrast optical microscopy. The method to measure total β-galactosidase activity was as described previously (21).

Densitometry

The density of blue coloration of individual cells developed with the X-gal *in situ* assay (Fig. 2B) was determined using an IS 1000 α-imager (Canbera Packard Inc.). For each photograph, which represented a different time point (Fig. 1), the density of 90–100 randomly chosen cells was determined and from this the background determined from areas of the photograph without cells was subtracted. The most intensely colored cells from all photographs were arbitrarily given a value of 10, while the least intense (unstained) cells were given a value of 0. The intensity of other cells was scored between 0 and 10, where 0 means the cell had a relative intensity of <1, 1 means the cell had a relative intensity of between 1 and 2, 2 means the cell had a relative intensity between 2 and 3, and so on.

Table 1. Analysis of β-galactosidase expression and X-gal staining in transgenic mice

Line[a]	Copy number[b]	Staining cells[c] (%)	Expression[d] (mU/mg)	Exp./staining cell (mU/mg)/% cell
βA1 (l)	2	0	0	–
βA2 (l)	3	46	220	4.8
βA3 (l)	~200	0	0	–
βA4 (l)	5	58	270	4.7
βA5 (l)	1	28	60	2.1
βB1 (l)	3	38	25	0.66
βB3 (l)	14	80	590	7.4
βB4 (l)	3	87	720	8.3
βB5 (l)	4	82	610	7.4
βB6 (l)	3	84	900	11
βB7 (l)	7	11	8	0.73
βC1 (l)	20	89	1650	18
βC2 (l)	10	75	1020	14
βC3 (l)	16	35	105	3.0
βD1	12	48	1060	22
βD2	25	81	3900	48
βD3	50	70	2750	39
βD4	3	49	125	2.6
βD5	3	78	130	1.7
βD6	5	11	15	1.4
βD7	12	22	30	1.4
βD8	5	49	750	15
βD9	1	3	3	1.0
βD10	15	72	300	4.2
βD11	2	31	300	9.7
βD12	1	1	2	2.0
βD13	30	4	5	1.3
βD14	20	64	50	0.78
βD15 (l)	20	11	12	1.1
βD16 (l)	30	9	23	2.6
βD17 (l)	14	81	1020	13
βE1	5	22	10	0.45
βE2	4	5	6	1.2
βE3	0.6	25	9	0.36
βE4	4	14	6	0.43
βE5	2	5	1	0.20
βE6	2	82	120	1.5
βE7	1	15	8	0.53
βE8	1	43	50	1.2
βE9	5	1	1	1.0
βE10	0.7	29	25	0.86

[a](l) depicts bred lines. The other mice were analysed as founder fetuses 15 days following microinjection.
[b]Copy number was measured from F1 tail DNA for bred lines and from fetal liver DNA for founder fetuses.
[c]Cells from the fetal liver were stained with X-Gal for β-galactosidase and blue cells were counted after 24 h.
[d]β-galactosidase activity measured in protein extracts from fetal liver.

Methylation assays

DNA was assayed for methylation as described previously (32). Details are given in the legend to Figure 4. Note the different lanes in Figure 4 were all from the same Southern blot which was exposed to film for different times to even out the intensity of the individual lanes.

RESULTS

β-galactosidase expression is graded in transgenic mice carrying the *lacZ* gene under the control of the β-globin LCR

Although the β-globin LCR directs position-independent expression onto globin transgenes in mice (33), transgenes containing the β-globin LCR and the bacterial *lacZ* gene linked to the β-globin promoter are prone to strong position effects in mice (21). Among 42 transgenic samples studied, the level of *lacZ* transgene expression, as measured both at the mRNA and β-galactosidase activity level, varied >700-fold on a per copy basis between those mice that had detectable expression, and two samples gave no expression at all. Fetal liver cells from 41 of these transgenic animals, which included 15 bred lines, were dispersed into solution and were stained for β-galactosidase activity using the X-gal reagent (34). The percent cells that colored blue after a 24 h incubation, after which time no further staining was observed, varied widely among the different transgenic animals (Table 1). A low of ~1%, to a high of >85% was obtained, excluding the two samples that had no detectable expression at all. Greater than 80% apparently β-galactosidase positive cells likely represented all of the red blood cells, since fetal liver also contains hepatic cells which would not be expected to express the transgene as it is erythroid-specific (35).

The variation in the fraction of staining cells in the different transgenic animals was not due to a lack of precision in the assay since fetal livers derived from animals of the same transgenic line gave very little difference in the percent staining cells (not shown). It is possible that some of the variation between samples from founders was due to the presence of mosaic animals. If a founder was mosaic, some of the red blood cells may not have contained the transgene. However, a >10-fold variation in the fraction of staining cells was also observed between the different bred lines (Table 1). Since the transgene must have passed through the germ line for it to be transferred to offspring, all red blood cells in these lines must have contained the transgene. Therefore, a kinetic analysis of the *in situ* β-galactosidase assay was done to gain an appreciation of the mechanism of this heterocellular staining-position effect phenomenon.

Transgenic fetal liver samples derived from bred lines that showed either a relatively low (βD15, 11%), medium (βA5, 28%) or high (βB5, 82%) proportion of staining cells were viewed at different times of incubation with X-gal (Fig. 1). Note that all the other lines and founders were studied similarly and the results presented and discussed below were typical.

The binary model predicts that when comparing cells within a single transgenic sample the expressing cells should have approximately the same content of β-galactosidase enzyme. Thus, one would expect there would be little variation in the staining intensity of individual cells in a given sample. Indeed, this was seen to be the case for a *lacZ* transgene under the control of α-globin regulatory sequences in transgenic mice (13). However, in all of our samples the number of cells that could be

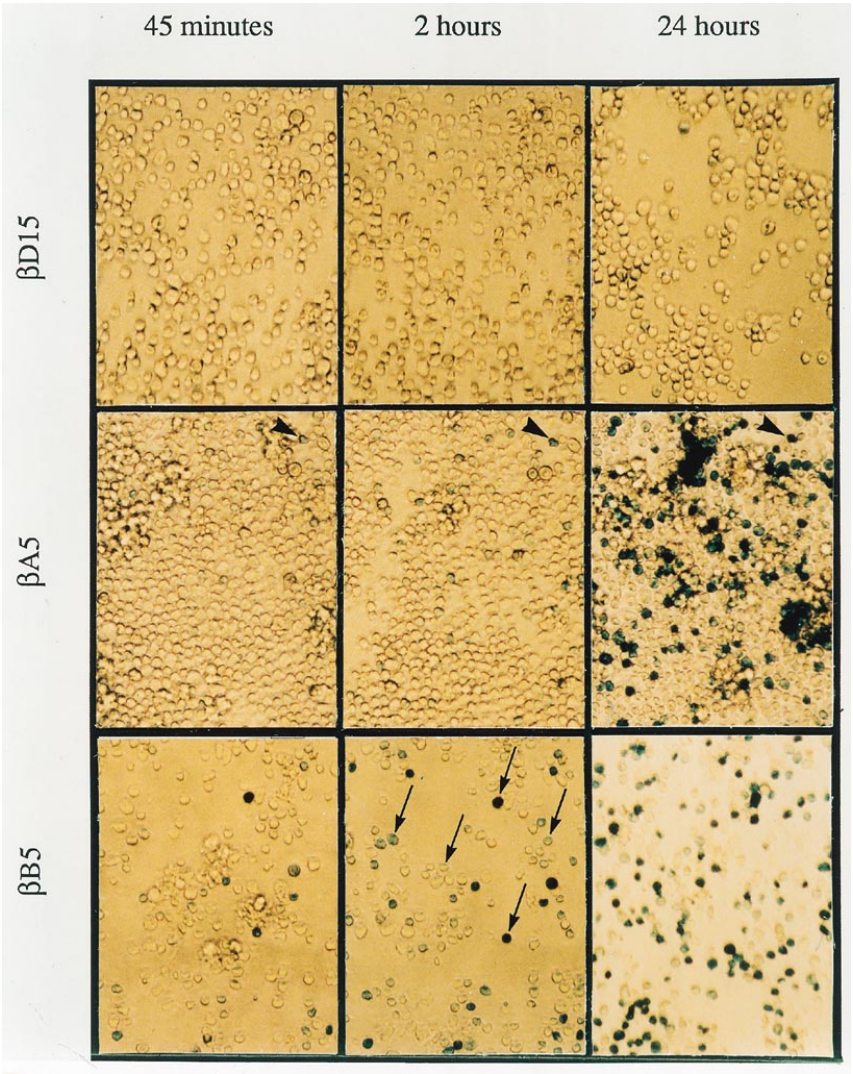

Figure 1. Kinetics of appearance of stained cells *in situ*. Fetal liver cells from transgenic lines that gave a low (βD15), medium (βA5) or high (βB5) fraction of staining cells were incubated with X-gal and were photographed at different times. The transgenic lines studied are indicated to the left of the rows, while the time of incubation is indicated at the top of the columns.

discerned to contain blue coloration increased with the time of incubation with X-gal (Figs 1 and 2A), suggesting that cells within a single sample did not contain the same amounts of β-galactosidase protein. This conclusion was supported by the observation that there was also a large variation in the intensity of staining of individual cells at any given time. This could be seen visually (see arrows in Fig. 1 for example, but note that the variation in cell intensity can be easily seen in all samples in Fig. 1 and was seen with every transgenic sample) and by determining the staining intensity of individual cells using densitometry (Fig. 2B). Note also that cells could be seen to increase their intensity of staining with the time of incubation (see arrow heads in Fig. 1), as would be expected if the intensity of blue coloration versus time reflected the level of β-galactosidase enzyme in individual cells. Thus, cells within each transgenic sample were not expressing at a set level as the binary model would suggest and as has been seen in other studies (13). Rather, there was a gradient in β-galactosidase activity.

To ensure the cell to cell variation in staining intensity within a single transgenic sample was not due to an incubation phenomenon, such as diffusion of β-galactosidase enzyme or reaction product from a positive cell to a negative cell, we mixed, in varying proportions, fetal liver cells from a transgenic animal that showed a high percent of positive cells with fetal liver cells from a non-transgenic animal. In all cases, the percent cells that could be detected as β-galactosidase positive was predictable based on the proportion of transgenic sample in the mix (results not shown). It also seemed possible that the cell to cell variation could at least partially reflect the changing differentiation status of developing erythroid cells in fetal liver. However, when fetal blood cells, all of which should be mature cells, were stained and looked at at different times they showed a similar gradient in staining intensity (results not shown). Hence, the differentiation status was playing a minor role if any. We have also previously shown that the level of β-galactosidase activity correlates with the level of *lacZ* mRNA (21). Thus, the results taken together suggested that the variation in

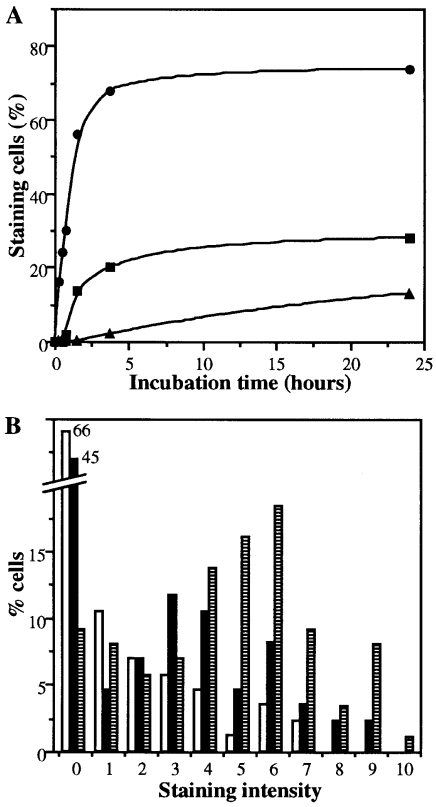

Figure 2. The fraction of detectable cells and the intensity of blue coloration increases with time. (**A**) Fetal liver cells from different transgenic lines (βB5, circles; βA5, squares; and βD15, triangles) were treated with X-gal and the percent cells that could be discerned to contain blue coloration was determined at different times. (**B**) Fetal liver cells of an animal from the βB5 line were treated with X-gal and the intensity of blue coloration of individual cells at 45 min (empty boxes), 2 h (filled boxes) and 24 h (dashed boxes) was determined using densitometry as indicated in Materials and Methods.

staining intensity reflected different levels of transgene expression in individual red blood cells.

The binary model also suggests that the level of expression per positive cell should be the same between different transgenic samples as, again, was observed to be the case for the *lacZ* gene in *cis* with the α-globin regulatory sequences (13). However, this was not the case for our transgenic samples. For example, in a sample that gave a low percent of detectable cells (top row in Fig. 1), stained cells could only barely be detected after 2 h of incubation. In fact, even after 24 h of incubation, none of the cells had attained an intense blue color. Rather, they were just detectable. In contrast, a transgenic sample with a high fraction of detectable cells (bottom row in Fig. 1) gave intense staining cells even after only 2 h of incubation, and many stained cells were already easily discernible after only 45 min of incubation. A transgenic sample that gave a medium proportion of apparent β-galactosidase positive cells (middle row in Fig. 1; compare with top and bottom rows) gave a profile of staining, in terms of the time blue cells first appeared and the average intensity of staining at any given time, that was between that for the low and high expressing samples. These results suggest that the average amount of β-galactosidase per expressing cell was varying considerably between the different lines. In fact, it appeared that samples that showed a low fraction of positive cells had less β-galactosidase enzyme per cell, on average, than samples that had a higher proportion of positive cells. To test these possibilities more rigorously, we calculated the amount of β-galactosidase activity per expressing cell for each sample and plotted the values against the total expression measured in extracts. With the binary model, since expression per positive cell should be constant between the different samples, one would predict the curve shown in Figure 3A. However, as can be seen in Figure 3B, the average level of expression per positive cell we observed was not constant. Rather it increased proportionally to the total expression (R = 0.981). Thus, not only was there a grading of expression between cells within a given sample, there was also a grading of expression when comparing cells between different samples. It is also interesting to note that the maximum level of β-galactosidase enzyme that cells could possibly produce was not reached since the curve in Figure 3B did not plateau at the higher values of expression per cell. Another manner of looking at the results is shown in Figure 3C. As the average level of expression per positive cell increases so does the number of cells that can be detected as positive. These two values are approximately proportional at the lower part of the curve and then plateau at >80% positive cells, which likely represents the total red cell population. This agrees with the finding that samples with a low fraction of positive cells give rise to less intense stained cells than samples with a higher fraction of positive cells (Fig. 1). The data in Figure 3C clearly indicate that it is the average level of expression per cell in the population of cells that determines the fraction of cells that can be detected as positive.

In presenting the binary model the effect of a variation in transgene copy number was not discussed (5). In any case, we have shown previously that expression of the *lacZ* gene in the transgenic samples studied here shows absolutely no relationship to copy number in terms of total β-galactosidase activity or mRNA levels in fetal liver (21). Moreover, there is also no relationship to transgene copy number when expression, in terms of percent cells stained or expression per cell, is looked at on an individual cell basis (Table 1). For example, a single copy transgenic can give more intensely stained cells (i.e. higher expression per cell) than a multi-copy transgenic (compare the 1 copy βA5 line with the 20 copy βD15 line in Fig. 1). Thus, transgene copy number does not have any bearing on the interpretation of the data presented here.

Gene silencing, as measured by methylation of the transgene DNA, was absent in samples that had detectable β-galactosidase activity

In vertebrates, DNA methylation at CpG dinucleotides is associated with gene silencing, although whether this is a direct cause of gene inactivity is not resolved (reviewed in 36,37). Since in many of our transgenic samples a large proportion of cells appeared to contain no detectable β-galactosidase activity, we wondered if this might have been due to direct silencing of the transgene in a proportion of cells. Therefore, we looked at the methylation status of the *lacZ* gene in fetal liver DNA by differential digestion with methylation insensitive (*Msp*I) and methylation sensitive (*Hpa*II) restriction enzymes. As can be seen in Figure 4, in transgenic lines with a relatively low (βA5, 28% and βB1, 38%) or a relatively high (βB5, 82% and βB6, 84%) fraction of staining cells, the *lacZ* transgene was not at all methylated. It was cut equally well and to near completion by both enzymes. On the other hand, in the two samples (βA1 and βA3) that gave no detectable β-galactosidase expression

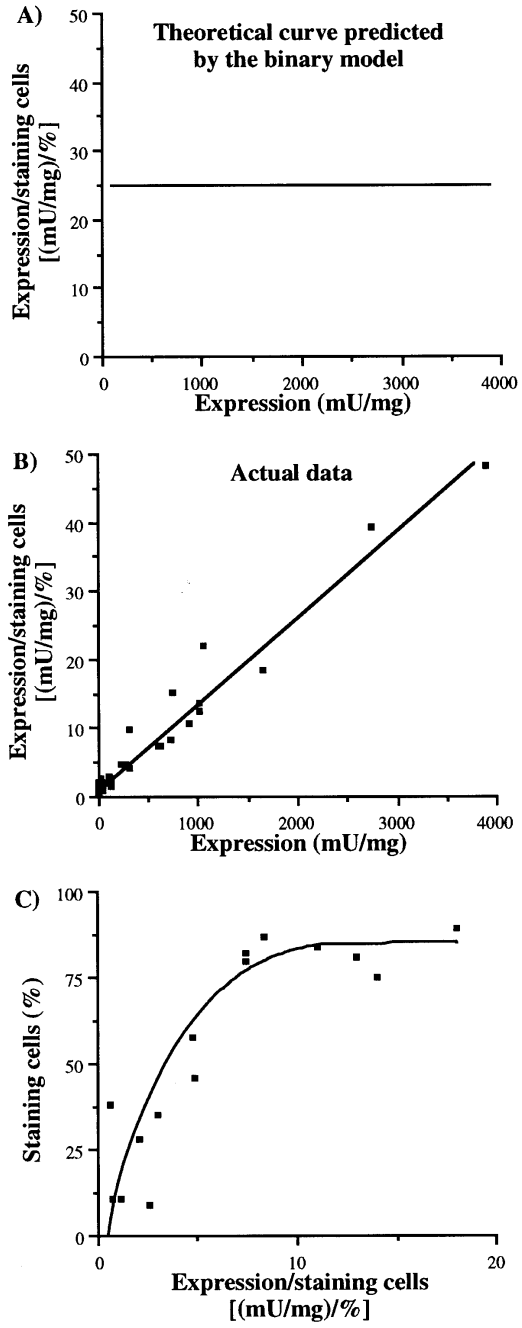

Figure 3. The average expression per cell determines both the fraction of cells that can be detected as β-galactosidase positive and the total level of β-galactosidase activity in the cell population. (**A**) The relationship between expression per staining cell and total expression expected for a binary model is shown. A straight line with zero slope is expected since the model predicts that all integration sites will give the same expression per cell and only the number of cells expressing should vary. (**B**) The actual data obtained are plotted. It can be seen that the expression per staining cell actually increases proportionally to the total expression. All transgenic samples, including lines and founders, but excluding the two transgenic lines that had no detectable expression, were used for this plot. (**C**) The fraction of cells that were β-galactosidase positive for each of the lines that had detectable expression is plotted against the average expression per positive cell. It can be seen that the number of cells that are detectable as β-galactosidase positive is determined by the average level of expression per cell. Note that founder transgenic samples were not used for the plot in (C), since the possibility that a founder was mosaic could have resulted in a fraction of the cells not containing the transgene, which would specifically affect the percent cells staining parameter.

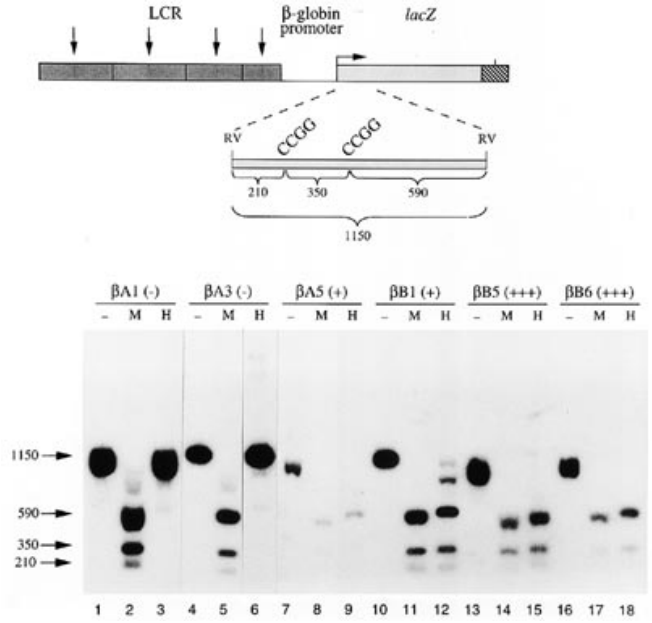

Figure 4. Methylation of the *lacZ* transgene DNA in different transgenic lines. Genomic DNA isolated from fetal liver was digested with *Eco*RV, which generates a 1150 bp fragment from the 5′ end of the *lacZ* reporter gene (top). The DNA was either not treated further (lanes marked –) or was digested with *Msp*I (lanes marked M) or *Hpa*II (lanes marked H). Both of these enzymes can restrict the sequence CCGG and will generate fragments of 590, 350 and 210 bp from the 1150 bp *Eco*RV fragment (see top and the positions of the fragments on the Southern blot are indicated by the arrows to the left). However, *Hpa*II is sensitive to methylation of the internal C residue and will not restrict the DNA if it is methylated, whereas *Msp*I is not sensitive to methylation here. The DNA was separated on agarose gels, transferred to nylon membrane and probed with ^{32}P-labeled *Eco*RV *lacZ* fragment. The transgenic line studied is indicated on the top of each triplet of lanes and the fraction of cells for each line that were β-galactosidase positive are indicated in brackets as –, no positive cells; +, a low fraction of positive cells; +++, a high fraction of positive cells. Note that the different lanes were spliced from autorads in which the same Southern blot was exposed to film for different periods of time to equalize the signal intensities.

at all (no detectable activity in extracts and no detectable positive cells), the *lacZ* transgene was completely methylated, as it was only digested by the methylation insensitive enzyme (Fig. 4, compare lane 2 with lane 3 and lane 5 with lane 6). Thus, in transgenic samples in which absolutely no *lacZ* transgene activity could be detected, it appeared that this involved silencing of the transgene in the total cell population. On the other hand, when the *lacZ* transgene was expressed in only a fraction of the cells, this was not a result of the *lacZ* gene being preferentially silenced in the cells that did not express the transgene. It is also worthwhile to note that Garrick *et al.* (38) also found that a *lacZ* transgene remained unmethylated in mice whether it was expressed at a relatively low or high level. However, these authors did not study lines in which the transgene was totally silenced, which we show here is associated with methylation.

DISCUSSION

In the results presented here, it was found that a *lacZ* transgene in *cis* with the β-globin LCR is expressed in a graded fashion on a cell to cell basis, both between cells within a single transgenic sample (Figs 1 and 2) and between different transgenic samples (Fig. 3B). As far as we are aware, this is the first demonstration of definite cell to cell gradation of transgene expression in mice, although it has

been documented in yeast studies previously (15). The graded cell to cell variation of expression within a single transgenic sample would not appear to be a position effect phenomenon *per se*, as it was observed in all 39 expressing transgenic samples studied. Therefore, this gradation occurs independent of the integration site. Nor does this gradient in expression appear to be something that is inherent to the *lacZ* transgene, since it did not occur in mice carrying the *lacZ* transgene controlled by α-globin regulatory sequences (13). Thus, we must conclude that this gradation in expression between cells is something that is inherent to the regulatory regions directing expression of the *lacZ* transgene in our mice, namely the LCR enhancers in conjunction with the β-globin promoter.

The position effects in our mice provoked a variable level of total expression in the population of red blood cells from one transgenic line to the next, with no relationship to copy number (21), and here we observed that in many transgenic samples, including bred lines, only a fraction of the red blood cells contained β-galactosidase activity. If this was primarily due to a binary mechanism controlling the position effects, we would have expected that the level of expression per expressing cell would be constant and that the total level of expression in the population would simply be determined by the fraction of expressing cells. However, the average level of expression per positive cell was not constant between the different transgenic samples, but increased proportionally to the total level of expression in the population (Fig. 3B). Thus, the position effect, in this case, determined the average level of expression per cell in the population. We refer to this as a graded model for position effects, as it describes both the gradation in the average level of expression per cell between different transgene integration sites and the cell to cell gradation in individual transgenic samples.

Since position effects are presumed to reflect the level of functioning of enhancers controlling the transgene, our results argue that the LCR enhancers do not work by a simple binary mechanism, for example, acting to prevent silencing of the gene in individual cells. Rather the LCR enhancers act to increase the average level of expression in individual cells. This conclusion contrasts with those of others (5,13) whose results have indicated that enhancers work via a binary mechanism. However, regulatory sequences different from those being studied here were investigated by these researchers. Thus, we suggest that the β-globin LCR enhancers work by the graded mechanism as we have described, whereas some enhancers may work via the binary model.

If the LCR enhancers act to increase expression in individual cells, why then do we observe heterocellular staining in some transgenic samples? There are at least two possible explanations. One would be that in a certain fraction of cells the transgene is silenced as the binary model would predict (5). However, we do not prefer this explanation, as we found that there is no methylation of the *lacZ* transgene DNA in samples that had detectable expression in only a proportion of the cells (Fig. 4). Moreover, in studies of yeast it was found that isolated non-expressing cells can revert back to expressing cells after several generations (15), suggesting that the transgene in non-expressing cells is not irreversibly silenced in such mixed populations. Still, we cannot rule out the possibility of gene silencing in a sub-population of the non-expressing cells in our case, and it should be realized that methylation of DNA may represent only the final stage of gene silencing. On the other hand, the heterocellular staining we observe in some samples could be related to the graded model. When a cell expresses the product of a gene, it must initiate transcription, elongate the transcript, process the mRNA precursor, transport the mRNA from the nucleus to the cytoplasm and translate the mRNA into protein. Moreover, enough protein must accumulate within the cell to form enough active enzyme in the case where gene expression is being measured at the enzyme activity level. The steady state level of mRNA and protein within a cell is a net result of synthesis and degradation at each of the steps. The graded model for position effects in combination with the random distribution of expression within individual cells in the population, would predict that a transgene strongly repressed by its position in the genome can fall below the level of actually making active protein or even initiating transcription in many cells (we use the term repression to mean a more dynamic state of gene inactivity, while the term silencing is meant as a more irreversible state of gene inactivity). The more strongly the transgene is repressed by its genomic position the higher the likelihood that a large fraction of cells will fall below the detection limit of the assay being employed to measure expression and this is because there is a cell to cell variation in expression levels (refer to Fig. 5). On the other hand, when the transgene is expressed at higher levels because of a more favorable genomic position, more, and possibly all cells will be above the detection limits of the assay (Fig. 5). Therefore, the graded model predicts that as the average level of expression increases in the population of cells so does the fraction of cells that express the transgene. This will plateau when all the cells can be detected as positive, as was seen to be the case here (Fig. 3C). Hence, the graded model explains why a fraction of cells do not express the transgene in samples with a low overall activity without invoking gene silencing.

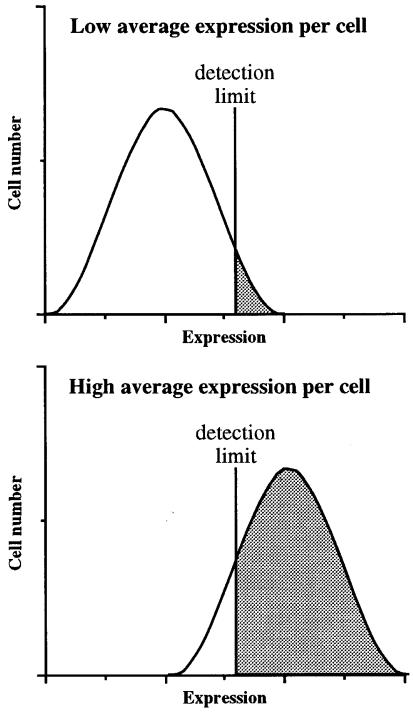

Figure 5. Theoretical curves showing that when there is a random cell to cell distribution of expression in a population of cells, a population with a low average level of expression per cell will have a much higher fraction of cells falling below the detection limit of the assay being employed [and many cells may not have any expression at all (see text)] than a population with a high average expression per cell.

At this point we can only speculate as to how the graded mechanism functions at the molecular level and how the β-globin LCR in conjunction with globin genes acts to prevent position effects. It has been suggested that the total level a globin gene is expressed in a cell is determined by the total time that gene is transcribed which, in turn, is dictated by the time it spends interacting with the LCR (27). If we can relate position effects to the amount of time a gene is transcribed and find a way to make it so that the β-globin LCR can confer position-independent expression onto the *lacZ* gene such that we can look at expression in individual cells, we should be able to address the mechanisms involved more directly in the future.

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