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Enhancers increase the probability but not the level of gene expression

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ABSTRACT **We have studied enhancer function in transient and stable expression assays in mammalian cells by using systems that distinguish expressing from nonexpressing cells. When expression is studied in this way, enhancers are found to increase the probability of a construct being active but not the level of expression per template. In stably integrated constructs, large differences in expression level are observed but these are not related to the presence of an enhancer. Together with earlier studies, these results suggest that enhancers act to affect a binary (on/off) switch in transcriptional activity. Although this idea challenges the widely accepted model of enhancer activity, it is consistent with much, if not all, experimental evidence on this subject. We hypothesize that enhancers act to increase the probability of forming a stably active template. When randomly integrated into the genome, enhancers may affect a metastable state of repression/activity, permitting expression in regions that would not permit activity of an isolated promoter.**

The mechanism by which enhancers activate transcription remains a subject for speculation. One proposed mode of enhancer action is to increase the rate of transcription from a linked promoter (Fig. 1*A*). This "rate" model is based on nuclear run-on assays of populations of cells transiently transfected with plasmid constructs, which found that more nascent transcripts were synthesized by cells if the transfected construct contained the simian virus 40 (SV40) enhancer (1, 2). The interpretation of this result as an increase in the rate of transcription in every transfected nucleus, or an increase in the density of RNA polymerase on each template (3, 4), underlies the assessment of much subsequent work on transcriptional control. A "probability" model of enhancer action (Fig. 1*B*) has been suggested by experiments that have examined single cells, rather than populations, expressing transfected constructs (5–7). These experiments revealed that enhancers increase the number of expressing cells but not the level of expression per expressing cell.

The results of the nuclear run-on experiments cited above are actually consistent with both models, since either an increase in the number of expressing cells in the population or an increase in the polymerase density per individual template would yield an increase in nascent transcripts in the total cell population. However, only the probability model is consistent with the single cell experiments.

We have made a detailed examination of the enhancer effect on expression of a linked reporter gene using both transient and stable expression assays. These experiments were designed to directly test the two models by distinguishing and separating expressing from nonexpressing transfected cells. We find that in both transient and stable assays a linked enhancer increases the number of cells actively expressing a reporter but not the amount of expression in active cells. In stably transfected clones, differences in expression levels are found, but these are not related to either the number of integrated copies of the construct or the presence of an enhancer. These results strongly favor a model in which enhancers act to increase the probability of a template achieving an active state rather than increasing the level of activity per template.

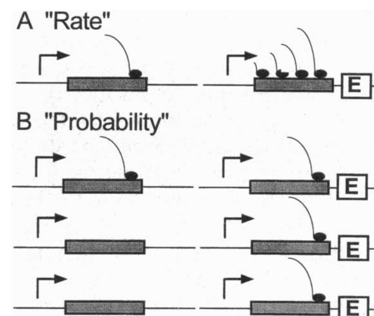

FIG. 1. The rate and probability models of enhancer action. (*A*) In the rate model, an enhancer (E) increases the density of polymerases over the transcription unit. (*B*) In the probability model, an enhancer increases the number of templates recruited, but there is no increase in polymerase density over a transcribed sequence.

MATERIALS AND METHODS

Construction of LacZ and β-geo Plasmids. Plasmids were constructed by standard methods. β-geo was excised from the plasmid pSAβgeo (8). The SV40 enhancer from bases 39–285, amplified by PCR with *Sal* I and *Bgl* II ends; the 1-kb *Sma* I/*Bgl* II fragment of 5′-HS2; and a 1.2-kb fragment containing the chicken 5′-HS4 element (provided by E. Epner, FHCRC) were cloned 3′ of β-geo. TK/LacZ/SVE was made by ligating a 252-bp fragment of the herpes simplex virus thymidine kinase (HSV TK) promoter 5′ of β-geo/SV40, and then exchanging the *Cla* I/*Sal* I fragment of β-geo with the *Cla* I/*Sal* I fragment of LacZ from PSDK LacZ (9). The SV40 enhancer was removed to make TK/LacZ. All fragments synthesized by PCR were sequenced to confirm their fidelity.

Cell Culture and Transfection. Conditions for growth of HeLa and K562 cells were as described (10). K562 cells (in 0.5 ml of 20 mM Hepes, pH 7.05/137 mM NaCl/5 mM KCl/0.7 mM Na_2HPO_4/6 mM dextrose) were shocked at 300 V/cm and 500 μF on a Bio-Rad Gene Pulser. Forty-eight hours after electroporation, 25 μl of medium was assayed for human growth hormone (hGH) by radioimmunoassay (Nichols Insti-

Abbreviations: SV40, simian virus 40; HSV, herpes simplex virus; TK, thymidine kinase; hGH, human growth hormone; MUG, 4-methylumbelliferyl β-D-galactoside; β-Gal, β-galactosidase; FACS, fluorescence activated cell sorter; PI, propidium iodide; FDG, fluorescein di-β-D-galactopyranoside.

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tute), and cells were plated in soft agar containing Iscove's medium supplemented with 10% calf serum and 1 mg of G418 per ml; 0.5×10^6 HeLa cells were transfected by the calcium phosphate method (11); 48 hr after transfection cells were harvested for assay of β-galactosidase (β-Gal) expression (transient assay) or replated on 96-well plates in medium containing 0.5 mg of G418 per ml (stable assay). Twenty-five microliters of medium was assayed for hGH.

Determination of β-Gal Activity. The 4-methylumbelliferyl β-D-galactoside (MUG) assay was performed on bulk lysates of 1000–5000 cells in 96-well plates on a Dynatech fluorimeter as described (12). Fluorescence of each sample was measured in triplicate and mean activity was determined. Protein content of lysates was determined by the Bradford method, and β-Gal activity was corrected for protein content. Data from six β-Gal assays performed in this manner were pooled and mean activity relative to a reference sample was calculated.

Southern Blot Hybridization. Preparation of genomic DNA, restriction digests, gel electrophoresis, membrane transfers, and probe radiolabeling were performed by standard methods (11). Probes for Southern hybridizations were from the β-geo gene (900-bp *Bam*HI/*Cla* I fragment) and the human β-globin gene (920-bp *Bam*HI/*Eco*RI fragment). Single-copy integrants were identified by digestion of genomic DNA with *Eco*RI (which has a single site in the β-geo constructs), followed by Southern blot analysis with the upstream *Bam*HI/*Cla* I LacZ probe and a downstream *Eco*RI/*Xho* I β-geo probe. In this way, one- and two-copy integrants were identified; higher copy numbers were estimated by comparison of band intensity to single copy clones with a PhosphorImager (Molecular Dynamics).

Fluorescence-Activated Cell Sorter (FACS-Gal) Assays. FACS analysis was performed on a Vantage instrument (Becton Dickinson immunocytometry systems). The FACS-Gal assays were carried out as described (13, 14). We do not detect β-Gal activity (by MUG) in cells sorted as negative in the FACS-Gal analysis and longer incubation with the substrate does not increase the proportion of cells counted as positive (data not shown). For these reasons, we believe that virtually all cells actively expressing β-Gal are being detected and that this method permits an accurate assessment of the proportion of live cells expressing β-Gal as well as separation of expressing from nonexpressing cells.

RESULTS

Transient Transfection Assays. The strategy for our studies of enhancer function in transient expression assays is illustrated in Fig. 2*A*. Plasmids containing the HSV TK promoter upstream of LacZ were constructed with or without a downstream SV40 enhancer (5, 15, 16) and transfected into HeLa cells with a reference plasmid. Seventy-two hours after transfection, the cells were stained with propidium iodide (PI) and the β-Gal substrate fluorescein di-β-D-galactopyranoside (FDG) and subjected to FACS-Gal analysis (12–14). PI is excluded by live cells, and under the conditions we used FDG is capable of detecting <5 molecules of β-Gal per cell (14). Equal numbers of live cells and live β-Gal-positive cells were sorted from the two pools and assayed with MUG to quantitate β-Gal activity (12).

As expected, addition of the downstream SV40 enhancer to the TK LacZ construct results in a large (10-fold) increase in β-Gal activity in the total pool of live transfected cells (Fig. 3*A*). FACS analysis reveals (Fig. 3*C*) that the enhancer also stimulates an ≈10-fold increase in the proportion of cells expressing β-Gal. In these assay conditions, FACS-Gal reliably detects cells expressing even minute amounts of β-Gal (14) but does not accurately quantitate expression since in many cells the substrate concentration is rate-limiting. When expressing cells are sorted by FACS-Gal and then analyzed for β-Gal

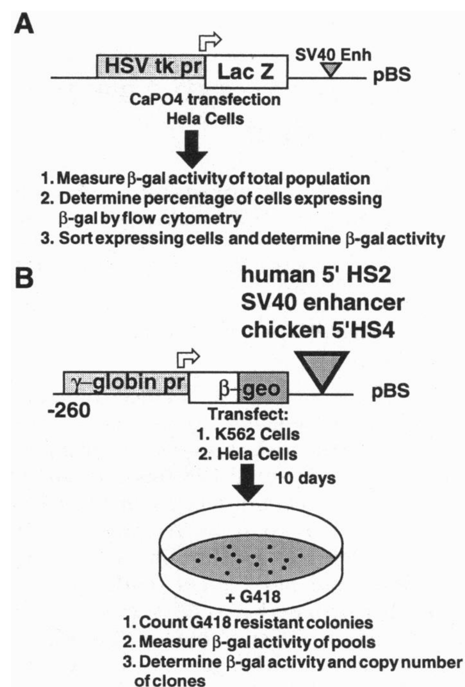

FIG. 2. (*A*) Schematic representation of transient transfection assays. Test plasmids containing the LacZ (β-Gal) reporter driven by the HSV TK promoter, with and without the SV40 enhancer, were cotransfected into HeLa cells with a hGH control plasmid to correct for transfection efficiency (17.5 μg of supercoiled test plasmid and 2.5 μg of hGH control plasmid). After transfection, live cells were collected by FACS on the basis of PI exclusion and assayed for β-Gal activity by MUG conversion. For each construct, the proportion of transfected cells that expressed β-Gal (as detected by fluorescence of the substrate FDG) was measured by FACS. In addition, transfected cells expressing β-Gal were sorted into pools by flow cytometry and the amount of β-Gal activity in these cells was determined by MUG conversion. (*B*) Schematic representation of stable expression assay. Linearized plasmids were transfected into HeLa cells (17.5 μg of test plasmid and 2.5 μg of hGH control plasmid) and the erythroleukemia cell line K562 (20 μg of test plasmid and 5 μg of hGH control plasmid). Test constructs contained the bifunctional reporter β-geo driven by the human $^{A}\gamma$-globin promoter (from −265 to +47) either alone or with the 5′-HS2 enhancer from the human β-globin LCR, the SV40 enhancer, or the chicken 5′-HS4 boundary element cloned downstream of the reporter. Forty-eight hours after transfection, cells were replated in medium containing G418; 10–14 days later, the number of G418-resistant colonies was counted and corrected for transfection efficiency with a hGH control plasmid. Several pools of 25 clones were created for each construct, and β-Gal activity (by MUG conversion) was determined for the pools. Finally, individual clones were isolated; copy number and β-Gal activity were determined for each clone. Data from stable transfections were pooled from three transfections performed in duplicate, and SEM was determined.

activity with the quantitative MUG assay, only a slight increase in the level of β-Gal expression is observed in the presence of the enhancer (Fig. 3*B*). If there were a threshold of β-Gal expression below which cells are not detected with FDG, we could be studying only those expressing relatively high levels of the reporter. It should thus be emphasized that in the transient assay, the increase in β-Gal activity in cells transfected with the enhancer construct is closely paralleled by an increase in the number of expressing cells detected by FACS-Gal (Fig. 3). As discussed above and in ref. 14, there is no detectable (by MUG) β-Gal activity outside the cells scored as positive in the FACS-Gal assay; thus, the increase in β-Gal activity in the transient assay must be entirely attributable to cells scored as positive by FACS-Gal. It is likely that there is only one or a few active template molecules in each cell; either model (rate or probability) would predict that multiple active templates would

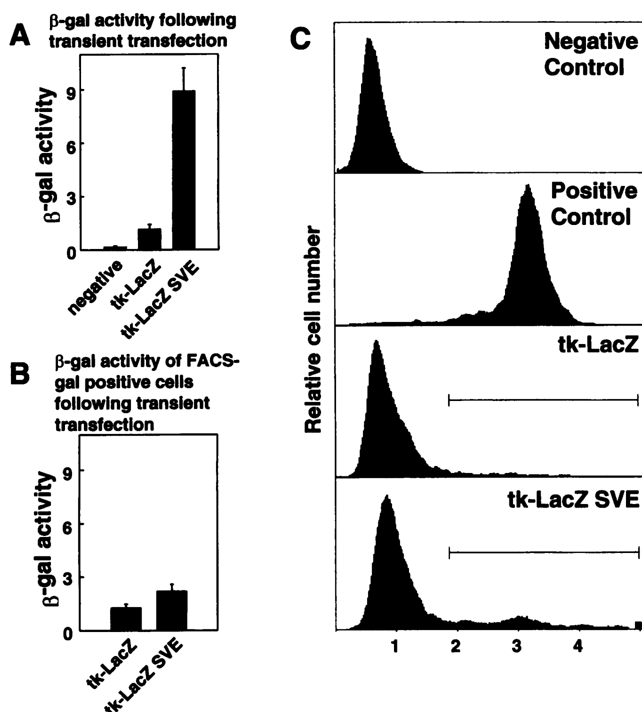

FIG. 3. Enhancer activity after transient transfection. Constructs with (tk-LacZ SVE) or without (tk-LacZ) the SV40 enhancer were transfected into HeLa cells. Forty-eight hours after transfection, cells were analyzed by FACS-Gal, and relative β-Gal level (by MUG conversion) and proportion of cells expressing β-Gal were determined after correction for transfection efficiency with hGH. After a 2-hr incubation on ice, cells were analyzed by FACS configured for fluorescein analysis. The fluorescence intensity of individual cells was measured as relative fluorescence units. Ten thousand live HeLa cells for each construct were collected on the basis of PI exclusion. Five hundred cells expressing β-Gal were collected after setting sort gates that included all β-Gal-expressing cells (defined as activity level exceeded by 0.1% of negative control HeLa cells). The MUG assay for β-Gal activity was performed on these cells. (*A*) Presence of the SV40 enhancer increased expression ≈10-fold in live transfected cells. β-Gal expression of mock-transfected HeLa cells is shown as a negative control. Data from four transient transfections were pooled and SEM was determined. (*B*) After transfection, HeLa cells expressing detectable levels of β-Gal as assayed by FACS-Gal were collected by flow cytometry (from the same populations as in *A*). All cells expressing β-Gal were collected for quantitation of β-Gal activity. Activity was quantitated from an identical number of β-Gal-expressing HeLa cells containing tk-LacZ and tk-LacZ SVE plasmids. As determined by MUG conversion, activity was only slightly increased in cells containing the tk-LacZ SVE plasmid compared to cells containing tk-LacZ. (*C*) FACS histograms of HeLa cells 48 hr after transfection with tk-LacZ and tk-LacZ SVE. The *y* axis denotes the number of cells and the *x* axis represents a 4-decade logarithmic scale of β-Gal expression. Negative control cells are mock-transfected HeLa cells, and positive control cells are HeLa cells stably transfected with a LacZ control plasmid. Histogram of cells transfected with tk-LacZ shows a small percentage of β-Gal-expressing cells. Fraction of expressing cells is increased 13-fold (after correction for transfection efficiency) when cells are transfected with tk-LacZ SVE (*Bottom*).

produce significantly higher reporter expression in cells containing the enhancer construct, and we do not observe this. In addition, Weintraub (6) reported results similar to ours under conditions designed to give only one template per cell.

Stable Transfection Assays. We studied expression from constructs stably integrated into the host cell genome with the strategy diagrammed in Fig. 2*B*. The reporter in these assays is β-geo, a fusion of the LacZ and neo^R genes (8). This reporter can be used to both select stably expressing cells and conveniently quantitate expression. We analyzed constructs in the colony assay, which measures the number of G418-resistant clones produced by transfection and drug selection of cultured cells (7, 10, 17, 18). Pools of G418-resistant clones with and without enhancers were expanded in G418. Mean expression levels in these pools was then quantitated by the MUG assay. Further analysis was performed by picking individual clones, expanding them in G418, and assaying β-Gal levels and the number of integrated copies of plasmid.

We constructed a series of plasmids and tested them in both the human erythroleukemia cell line K562 (19) and in HeLa cells by the strategy outlined above. In the γ/β-geo construct, the SV40 enhancer, 5′-HS2 from the human β-globin locus or 5′-HS4 from the chicken β-globin locus, was cloned downstream of β-geo (Fig. 2*C*). 5′-HS2 is a strong enhancer in erythroid cells (21, 22). Chicken 5′-HS4 was recently reported to disrupt interactions between flanking DNA sequences and promoters in both mammalian cells and *Drosophila* (19) and is thus an example of a boundary element whose activity is not limited by tissue or species. These constructs were used to determine whether enhancer activity is shared by a boundary element and whether the increase in colony numbers associated with the presence of an enhancer is associated with higher expression per integrated copy of plasmid.

In K562 cells, 5′-HS2 produces a 23-fold enhancement of colony numbers, but SV40 and 5′-HS4 have no effect (Fig. 4*A*). In HeLa cells, a 9-fold increase in colony numbers is produced by the SV40 enhancer, but 5′-HS2 and 5′-HS4 have no effect. Pools of colonies (5 pools of 25 colonies for each construct) were isolated from the experiment described in Fig. 4*A*. These pools were expanded and assayed for β-Gal activity with MUG. No differences are observed in the enhancer-containing and enhancerless pools in K562 cells, while in HeLa cells the enhancer pool demonstrates 3-fold lower activity (Fig. 4*B*). Thus, while an enhancer increases the likelihood that an integrated construct will be expressed, average expression

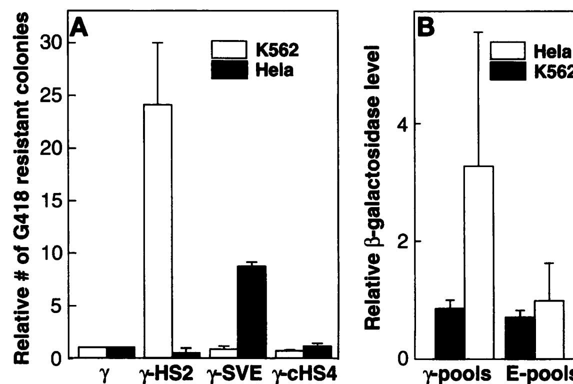

FIG. 4. Stable expression of β-geo in pooled cells. (*A*) Colony assay. Each of the constructs shown in Fig. 2*C* was transfected into HeLa and K562 cells, and G418-resistant colonies were counted. Relative numbers of K562 colonies (open bars) and HeLa colonies (solid bars) are shown after correction for transfection efficiency. In K562 cells, the 5′-HS2 enhancer increased the number of colonies by >20-fold, while the SV40 enhancer had no effect. Conversely, in HeLa cells, the SV40 enhancer increased the number of colonies by 9-fold, while the erythroid 5′-HS2 enhancer had no effect. The chicken 5′-HS4 boundary element had no apparent activity in either cell line. Data from colony assays were pooled from three transfections performed in duplicate and SEM was determined for each construct. (*B*) β-Gal activity in stably expressing K562 (solid bars) and HeLa (open bars) cells. Five pools of 25 G418-resistant colonies were created for γ/β-geo in both HeLa and K562 cell lines (γ-pools), and for γ/β-geo/HS2 in K562 cells and γ/β-geo/SV40 in HeLa cells (E-pools). β-Gal activity of cellular lysates from the pools was determined by MUG conversion, and relative activity is shown. Presence of an enhancer did not increase the level of expression in either HeLa or K562 cell lines.

levels in those cells having active integrated constructs do not show large differences.

Expression and Copy Number in Clones. Clones from the colony assays described above were analyzed for β-Gal expression and the number of copies of the β-geo construct. Significant variation in β-geo expression is observed among clones; this variation is not, however, related to the presence of an enhancer (Fig. 5). If an enhancer does confer a higher expression rate, two predictions can be made. First, transfection with enhancer constructs might produce more cells with sufficient expression to pass a threshold of expression required for G418 resistance. However, promoter-only cells above the threshold expression level would still have a lower average level of expression than enhancer-containing cells; we do not observe this despite broad variation in β-geo expression in our clones (Figs. 4 and 5). Second, fewer integrated copies of the enhancer construct should be required to reach a threshold for G418 resistance (assuming a correlation between expression and copy number), with fewer copies in clones containing an enhancer. We observe neither correlation between expression and copy number nor fewer copies in clones containing an enhancer. Presumably, differences in expression are related to the sites of integration of the constructs. The absence of copy number dependence in the HS2 constructs is consistent with studies of this element in transgenic mice (21, 22).

DISCUSSION

A Binary Model of Gene Expression. The results presented above suggest that enhancers act to increase the probability of the initial establishment of an active template but not the rate of expression. Differences in expression of integrated constructs are not related to the presence of an enhancer and likely reflect genomic context. When considered with the absence of an enhancer effect on level of expression per template, these results imply that rates of transcription are set by factors acting over large regions of chromatin, as did earlier studies of the relationship between proviruses and adjacent host chromatin (23).

Our studies provide strong support for previous reports of an enhancer effect on probability rather than rate of transcription (5–7). In one, mutations in the SV40 enhancer decreased the percentage of cells expressing the T antigen (5). Weintraub (6) found that the SV40 enhancer increased the number of cells expressing a reporter, but that expressing cells have equivalent levels of expression whether or not an enhancer is present. Linkage of the 5′-HS2 enhancer to neo^R was found to increase the number of G418-resistant colonies but not the level of neo^R expression in resistant colonies, a result very similar to our own (7). While these reports have not been widely cited, together with the work presented here they make a strong case for a model in which a linked enhancer increases the likelihood of a promoter achieving a stable active state but not the rate of mRNA production. We term this mechanism "binary" because of its on/off nature. The many experiments using population rather than single cell assays do not permit distinction between the binary and graded modes of enhancer action and so are not useful in judging the relative merits of the two models. Considerable further evidence supporting the binary model has accumulated from various systems (12, 24–30). Interestingly, many of these results would have been interpreted as graded effects if the entire population of stimulated cells had been studied rather than single cells.

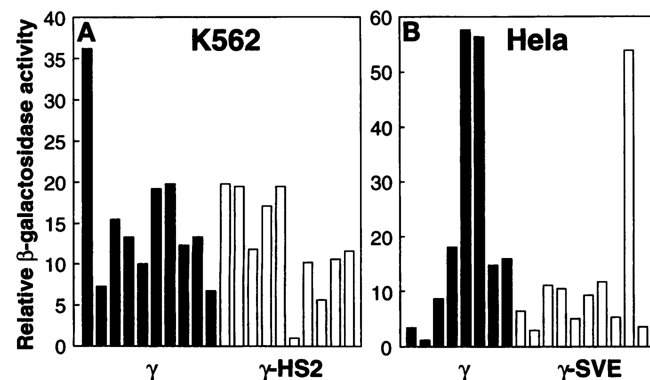

FIG. 5. Stable expression of β-geo in clones. Ten G418-resistant K562 clones containing γ-βGeo and 10 containing γ-βGeo HS2 (γ-HS2) (*A*) and 8 G418-resistant HeLa cell clones containing γ-βGeo and 10 containing γ-βGeo SV40 enhancer (γ-SVE) (*B*) were expanded and relative β-Gal activity was determined by MUG conversion. Copy numbers of clones were estimated by Southern blot analysis (data not shown) and the average β-Gal activity of each clone was corrected for copy number. Neither construct demonstrates expression that is copy number dependent. The clones do demonstrate marked variation in β-Gal activity, but the presence of either the HS2 or the SV40 enhancer does not increase the activity.

Enhancers May Facilitate the Formation of Stable Chromatin Domains. We propose that enhancers facilitate the formation of stable domains within which promoter activity is permitted. The simplest interpretation of our results is that the enhancer effect in the transient and stable assays is the same: to increase the probability of achieving a stable active transcriptional state. However, the situation in the stable assay may be more complex.

Integration may occur randomly into chromatin that varies in its ability to allow transcriptional activity (31). Constructs may be relatively more efficient at creating an active region within a region of inactive chromatin when they contain an enhancer. An enhancer would thus tend to increase the number of sites at which activity could occur after integration. This would account for the results of the colony assay. (The objection that the similar levels of expression seen in the stable assay could be due to the integration of enhancerless constructs near cellular enhancers is not supported by the transient expression assays, where the same effect occurs without integration.) Control of the integration site will be required to investigate this issue more thoroughly.

In their normal context, different genes are expressed at different levels (3, 4) and must contain all of the elements necessary to ensure expression in appropriate cell types. The experiments presented above suggest that the cis-acting control elements (enhancers and promoters) function to activate transcription but that other factors, particularly the chromosomal context, may determine the rate at which a gene is expressed. Clearly, additional experiments addressing this issue are required for a more detailed understanding of the mechanisms underlying control of the rate and developmental timing of transcription.

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