Transcriptional enhancers act in cis to suppress position-effect variegation

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We have examined the basis of enhancer effects on gene expression by altering the action of enhancers on expression of a stably integrated reporter gene. We used two distinct experimental approaches: recombinase-mediated deletion of an enhancer and modulation of the activity of another enhancer composed of downstream metal response elements (MREs). The IAP recombinase was used to delete the 5'HS2 globin enhancer from a site downstream of β-geo at nine separate integration sites in K562 erythroleukemia cells. In no case does deletion of 5'HS2 have a significant effect on the level of expression; however, the deletion does increase dramatically the rate at which expression of β-geo is silenced. Zinc stimulation of a metallothionein enhancer has no effect on the level of reporter expression, but slows the rate of silencing. Silencing in both cases is highly site dependent, and resembles position-effect variegation (PEV). These results strongly support a binary mode of enhancer action, as in both cases the enhancer maintains reporter expression without a strong effect on the level of expression. Taken together, these findings suggest that transcriptional activators have a direct interaction with repressive chromatin structures, which is independent of an effect on the rate of transcription. We propose that cis-acting transcriptional control elements may act primarily through this mechanism.

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Two distinct modes of enhancer action on gene expression have been proposed: (1) graded, in which the enhancer increases the rate of transcription from a linked promoter (see Lewin 1990, Singer and Berg 1991), and (2) binary, in which it increases the probability that a promoter will achieve and maintain an active state (Walters et al. 1995 and references therein). The first of these models is based on studies that used transient transfection assays (Treisman and Maniatis 1985; Weber and Schaffner 1985). These studies found that when entire populations of transfected cells were assayed, there was an increase in the amount of RNA produced if an enhancer was included in the transfected construct. However, assays of populations of transfected cells do not distinguish graded and binary mechanisms of control, as either an increased number of active templates or increased activity of each template will result in an increase in the total product in a population of transfected cells. The binary model of RNA polymerase II enhancer action is supported by a small number of reports that have distinguished the proportion of expressing cells and the level of expression in expressing cells; these studies have found consistently that enhancers increase the number of expressing cells but not their level of expression (Moreau et al. 1981; Weintraub 1988; Moon and Ley 1990; Walters et al. 1995). Regulation of transcription by RNA polymerase I and III enhancers has long been thought to act by a binary mechanism (Brown 1984; Reeder 1984, 1992).

Stably integrated plasmids are likely to be subjected to influences from flanking chromatin structures that vary widely with the integration site; the usual result of these influences is silencing of gene expression. A small number of reports have suggested this effect on proviruses and transfected genes (Davies et al. 1982; Feinstein et al. 1982; Conklin and Groudine 1986), but more information is derived from studies of position-effect variegation (PEV) and related phenomena in Drosophila, yeast, and mice (Cattanach 1974; Henikoff 1992; Rivier and Rine 1992; Allshire et al. 1994; Pirrotta and Rastelli 1994; Tartof 1994). In those systems, transfer of a transcription unit to a site near a centromere, a telomere, the mating-type locus, or the X chromosome results in silencing of expression in a proportion of cells; the silenced state is stable and clonally heritable, and probably is attributable to the packaging of the affected DNA into stable heterochromatic structures (Karpen 1994). Transgenes in mice are subject to a similar effect (Robertson et al. 1995), although there the role of centromeres and telomeres has not been established; arrays of a construct may be foci

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for the formation of heterochromatin (Dorer and Henikoff 1994). The action of Polycomb Group [Pc-G] genes on the homeotic complexes in Drosophila has also been related to PEV (Chan et al. 1994; Pirrotta and Rastelli 1994; Orlando and Paro 1995; Paro 1995). Silencing in PEV is facilitated by a large number of protein factors, many of which are known to be components of chromatin, and antagonized by other proteins, some of which are transcriptional activators (Henikoff 1992; Tamkun et al. 1992, Faras et al. 1994). Little information is available on the interaction of cis-acting transcriptional control elements [promoters and enhancers] with repressive chromatin effects, but in one system an active promoter has been shown to counteract telomeric position effect (Aparicio and Gottschling 1994). The binary action of enhancers on promoters is consistent with the view that they counteract silencing. Much evidence supports the view that enhancers and promoters are foci for changes in chromatin structure, although the nature and consequences of these changes are less clear (Forrester et al. 1986; Pikaart et al. 1992; Jenuwein et al. 1993).

In earlier experiments, we examined the basis of the positive effect of the SV40 and β-globin 5'HS2 enhancers on gene expression, and found that they increase greatly the probability of establishing expression but have no effect on the level of reporter gene expression [Walters et al. 1995]. In transient assays, this is reflected in a higher proportion of transfected cells expressing the reporter, in stable assays [colony assays] the effect is to produce a greater number of drug-resistant colonies. In neither assay do those cells actually expressing the reporter have higher average levels of expression when the transfected construct contains an enhancer. In stable assays there is a large variation in expression between clones that cannot be attributed to copy number. Although integration of enhancerless constructs near endogenous enhancers could explain these results, this view is not consistent with the results of transient assays, where no integration occurs. Thus, stably integrated plasmid constructs are subject to differences in expression, apparently caused by factors at the integration site ("position effects") that make direct comparison of different constructs difficult. Because integration may occur randomly into chromatin that varies in its ability to allow transcriptional activity, not all integration events result in an active construct (Davies et al. 1982; Feinstein et al. 1982, Conklin an Groudine 1986). We hypothesized that if an enhancer made a construct more efficient at creating an active region within a region of inactive chromatin, it would tend to increase the number of sites at which promoter activity could occur after integration. This would account for effects in colony assays, where enhancers produce more drug-resistant clones. The probability of establishing expression, as well as its level and maintenance, may be the result of an interplay between chromatin at the integration site and the control elements in the construct. An enhancer might permit activity in sites at which a promoter alone would be insufficient to do so, but at any integration site stability of expression would depend on the ability of the integrated elements to create and maintain a domain permissive for template activity. This model predicts that at a defined integration site expression would be more stable, but not higher, in the presence of an enhancer [i.e., removal of an enhancer or a protein functioning to activate it] would lead to silencing of expression rather than a reduction in its level.

We now describe studies of enhancer effect that control for the influence of the plasmid integration site by manipulating the activity of enhancers in stably integrated plasmid constructs. In these studies we have used a system that permits separation of effects on the level of expression from effects on the rate at which reporter expression is silenced. In a construct from which the 5'HS2 enhancer can be deleted by a site-specific recombinase, deletion of the enhancer has only a minor effect on the level of reporter expression, but results in silencing at a greatly increased rate in most integration sites. When an enhancer consisting of the metal response elements [MREs] of the metallothionein promoter is located downstream of the reporter gene, metal induction has no effect on level of expression but decreases the rate of silencing. These results are inconsistent with a strong enhancer effect on the rate of transcription from a promoter and suggest that transcriptional activation results in disruption, or prevention of the formation, of chromatin structures that silence expression. In aggregate, our results are compatible with a model in which transcriptional activators interact with repressive chromatin structures to permit promoter activity without a direct effect on the rate of transcription. An activator may increase transcription when bound near the site of initiation, but when bound at more distant sites only stabilize expression. We propose that the major function of cis-acting transcriptional control elements is to stabilize expression rather than to modulate the rate of transcription.

Results

Experimental strategy

In studies of expression from integrated constructs, we have used the β-geo reporter, a fusion protein with both β-gal and neoR activities [Friedrich and Soriano 1991], and a fluorescence-activated cell sorter assay, which gives a direct and highly sensitive measure of the proportion of cells in a population that are expressing β-galactosidase (FACS/Gal) [Nolan et al. 1988; Fiering et al. 1990, 1991; Walters et al. 1995]. β-geo permits both quantitation of the expression level of the drug resistance gene and study of its behavior in the absence of selection for expression. Our strategy has been to transfec transfect K562 cells with β-geo constructs, select G418-resistant clones, and characterize those with single integrated copies of the construct. Use of single-copy integrants eliminates problems created by activity of multiple copies in an array, and is necessary for the recombination step [see below]. Quantitation of expression by 4-methylumbelliferol-β-D-galactoside [MUG] conversion gives
Effects of enhancer action: (1) an increase in the level of reporter expression, and (2) an increase in the stability of f-geo expression. We have used this system to examine the effect of enhancers on expression level and the rate of silencing in two ways: by deleting an enhancer and by stimulating a metal-inducible enhancer.

Deletion of the 5'HS2 enhancer accelerates silencing

We constructed a plasmid ([gamma]/beta-geo/HS2-FRT) in which f-geo expression is driven by the [gamma]-globin promoter; a downstream 5'HS2 enhancer is flanked by flp recognition target [FRT] elements, which are targets for the flp recombinase [Fig. 1]. 5'HS2 is a component of the human [alpha]-globin locus control region [LCR] [Evans et al. 1990; Caterina et al. 1991]; it acts as a strong enhancer in both transient and stable expression assays in K562 erythroleukemia cells [Evans et al. 1990; Caterina et al. 1991; Chung et al. 1993; Walters et al. 1995]. Expression of flp in cells carrying this construct results in recombination between the two FRTs and deletion of the intervening enhancer [O'Gorman et al. 1991; Fiering et al. 1993; Kilby et al. 1993]. This strategy permits an assessment of reporter gene activity, in the presence and absence of an enhancer, that is controlled for integration position. We transfected the [gamma]/beta-geo/HS2-FRT construct into K562 cells and identified nine G418-resistant clones containing single unrearranged copies of the plasmid, integrated in different sites. These clones were then transfected with pflpHygro, a plasmid expressing both the flp recombinase and hygromycin resistance. Hygromycin-resistant clones were selected and screened by Southern blot analysis for loss of the enhancer [Fig. 1B]; neither parent nor hygromycin-resistant subclones were maintained in G418 during this step.

The level of f-geo expression and the rate at which it was silenced were compared in parent clones and their subclones lacking the 5'HS2 enhancer, maintained in the same conditions. Because cells were passaged without G418 selection, by the time screening for the enhancer deletion was completed they comprised a mixed population of reporter expressing cells and cells in which the reporter was not expressed. At the start of the analysis populations of f-gal-expressing cells were isolated from both parent clones and enhancer-deleted subclones by FACS/Gal and expanded in culture [FACS/Gal will detect cells with amounts of f-gal as low as 5 molecules per cell [Fiering et al. 1991]]. Assay of these populations by MUG conversion could give apparently low levels of expression if many cells are not expressing. To overcome this potential problem, the fraction of cells expressing f-gal in both parent and subclones was measured at intervals by FACS/Gal, and at each interval f-gal activity was quantitated by MUG conversion. The f-gal activity was then corrected for the percentage of cells expressing f-gal, and f-gal activity per expressing cell was determined. Using this method, we find only a slight (twofold or less) decline in expression level per cell after deletion of the enhancer; in some clones there is no decline [Fig. 2A].

In contrast to the slight effect on the level of expression, deletion of 5'HS2 results in a dramatic increase in the rate at which f-geo is silenced at many but not all integration sites [Fig. 2B]. We find that after removal of G418 selection, each clone reverts to a silent state at a characteristic rate. This rate is sufficiently slow in the parent clones that little silencing is evident over the period in which these clones were analyzed [Fig. 2B]. There is marked variation between sites in the rate of silencing after deletion of 5'HS2. The deleted subclone from line 24 remains nearly as stable as the parent line. Deleted subclones from the other lines are less stable than their parent lines. To eliminate the possibility of false positive cells from the sorting process biasing the stability assay, we used selection in G418 to expand populations of f-geo-expressing cells from these subclones and compared their stability of expression (after removal of G418 selection) with their parent lines treated in the same way. Subclones 12, 16, 19, 21, 30, and 45 are distinctly less stable than their parent lines; silencing of f-geo expression occurs over a period of weeks to months [Fig. 2B]. The result of this analysis is the same when expressing cells are sorted by FACS/Gal instead of expanded in G418 [not shown]. This finding may explain a previous observation that deletion of an enhancer from an integrated construct resulted in complete loss of expression, in that system there was no way to identify, recover, and study a small proportion of expressing cells [Grosschedl and Marx 1988]. It is significant that in line 24, in which expression is stable after deletion of 5'HS2, the level of f-geo expression declines only slightly (comparable to the twofold decline in other lines). This suggests that correction of the MUG assays for the proportion of expressing cells is not falsely elevating the apparent expression levels in the subclones; in line 24 there is no correction factor. Cells maintained in G418 do not show higher levels of f-gal activity than cells maintained in standard medium without selection [not shown].

f-geo expression was highly unstable in deleted subclones from two lines (lines 25 and 47). Cells from subclones of 25 do grow in G418, although much more slowly than the parent line. When removed from G418, they lose expression rapidly. We have been unable to expand the deleted subclone of 47 in G418. When maintained in selection for several weeks, live cells can always be found in the culture, and dead cells accumulate [not shown]. This finding is consistent with the interpretation that expression in these cells is so unstable that silencing occurs at nearly the same rate as expansion.

Stimulation of a metal-responsive enhancer retards silencing

As an independent approach to examine the conse-
Figure 1. Deletion of the 5' HS2 enhancer with flip recombinase. (A) The construct γ/β-geo/HS2-FRT contains β-geo driven by the human γ-globin promoter; a 1-kb fragment containing the 5'HS2 element from the human β-globin LCR is placed downstream between two flip recognition target (FRT) elements. Restriction sites used to generate probes for Southern blotting are marked: (R) EcoRI; (X) XhoI; (C) ClaI; (B) BamHI. Probes are denoted by solid lines beneath β-geo. The construct was electroporated into K562 human erythroleukemia cells and cells plated onto soft agar medium containing G418; resistant colonies were picked, expanded, and analyzed by Southern blot to identify clones carrying single unrearranged copies of the plasmid. Nine of these were transfected with a plasmid that coexpresses hygromycin resistance and the flip recombinase, and plated into medium containing hygromycin but lacking G418. Hygromycin-resistant colonies were screened for loss of the enhancer, and subcloned to ensure a pure population of cells carrying the deleted construct. Then, these clones were compared with the parent clones that had been maintained in the absence of G418. Expression levels were determined by FACS/Gal and MUG conversion. Stability of expression was assayed by creating pure populations of expressing cells (either by FACS sorting or growth in G418) and expanding these populations in the absence of G418. FACS/Gal was performed at intervals to measure the proportion of cells still expressing. (B) Southern blot of clones carrying the construct and subclones from which 5'HS2 was deleted. Genomic DNA was digested with EcoRI and probed with a 1.2-kb EcoRI-XhoI fragment 3' of the EcoRI site in β-geo. A unique integration site for each clone is demonstrated by the differences in size of the hybridization bands. Each subclone has a fragment ~1 kb smaller than that in its corresponding parent clone, reflecting loss of the 5'HS2 enhancer. Probing with a fragment of 5'HS2 demonstrates loss of hybridization to the subclones (not shown).

sequences of removal of enhancer activity from an integrated construct, we determined the effect of metal-inducible activating elements placed at a distance from the promoter on the rate and stability of β-geo expression [Fig. 3]. The mouse metallothionein (mMT-I) promoter responds to stimulation with heavy metals through the action of a single constitutively expressed factor MTF that binds to a series of six MREs arranged upstream of the TATA element [from −40 to −175] (Glanville et al. 1981; Stuart et al. 1984, 1985; Radtke et al. 1993). Binding of MTF to the MREs is induced by the presence of a number of heavy metals, and results in increased expression of metallothionein and of reporter genes driven by the mMT-I promoter (Mueller et al. 1988; Palmiter 1994). However, it is not known whether these effects represent increased expression in every cell. Earlier work had shown that the upstream region of the mMT-I promoter would activate heterologous promoters [Serfling et al. 1985; Stuart et al. 1985], and act as an inducible enhancer with a β-globin promoter when placed downstream of a chloramphenicol acetyltransferase [CAT] reporter [Serfling et al. 1985]. The enhancer effect was studied, however, in a transient assay in which no distinction between increased rate and increased numbers of active templates could be made [Serfling et al. 1985]. MREs placed in upstream promoter regions are able to increase the level of β-geo expression in K562 cells when stimulated with zinc [W. Magis and D.I.K. Martin, unpubl.].

The hypothesis that enhancer elements act on a binary switch in controlling gene expression predicts that in-
Enhancers suppress PEV


Figure 2. Level and stability of expression by clones with or without the 5'HS2 enhancer. (A) β-Galactosidase activity in parent clones (solid bars) and deleted subclones (open bars), brackets represent s.e.m. Values were calculated by correcting the β-galactosidase activity of cell lysates assayed by MUG conversion for the percentage of expressing cells assayed by FACS/Gal, and are averages of six separate determinations; they are normalized to the level in subclone 19. Clone 47 is not included in the figure because expressing cells could not be recovered after deletion of 5'HS2. (B) Stability of β-geo expression in parent clones (+) and subclones lacking the 5'HS2 enhancer (−) when expanded in the absence of G418 selection. The x-axis in these histograms represents a three-decade log scale of fluorescence. Histograms of K562 cells in the FACS/Gal assay show that deletion of 5'HS2 results in silencing of β-geo expression at an increased rate in all but one of the clones. Positive and negative controls are shown at the left. All cell populations were assayed at multiple time points, and deleted subclones showed an increasing proportion of silenced cells with time. The sequence of histograms labeled C25− represents the progression of silencing in the deleted subclone of C25 as an example; D denotes day. Because the clones differ in the rate at which they silence expression of β-geo, at later times some deleted subclones are silenced entirely, whereas at earlier points some show little silencing. The histograms shown are chosen to illustrate the difference between parent clones and their respective deleted subclones. Clones 12, 16, 21, and 30 were expanded without selection for 5 weeks. Clone 19 was expanded for 3 months, clone 45 for 4 months, and clone 24 for 5 months. Clone 25 was expanded for 4 weeks. The deleted subclone of 47 was silenced completely at the earliest time point analyzed, and expressing cells could not be expanded. Transfection of parent clones with a hygromycin vector and selection in hygromycin did not result in any change in the stability of β-geo expression (not shown).

duction of MREs at a distance from the promoter would not affect the level of β-geo activity but would retard the rate at which its expression is silenced. To measure the effect of metal induction on the level and epigenetic stability of reporter gene expression, we placed three copies of a 180-bp fragment containing the MREs from the metallothionein (MT) promoter downstream of the β-geo reporter [Fig. 3]. We derived a series of single-copy integrants of this plasmid in K562 cells and analyzed their response to zinc stimulation.

After characterization of clones carrying single-copy integrants, cells were maintained in G418 to ensure that all were expressing β-geo. The effect of zinc stimulation on the level of β-geo expression was then observed; 24 hr after zinc sulfate (80 μM) was added to the culture medium cell lysates were assayed for β-gal activity by MUG conversion and compared to lysates from unstimulated cells. In none of the γ/β-geo/MRE clones analyzed does zinc stimulation result in an increase in the level of β-gal expression [Fig. 4A]. This result is consistent with the binary model of enhancer action, which predicts that the MT enhancer would have no effect on the level of expression from templates that are already active. Differences in basal rate between individual clones are presumably attributable to characteristics of their integration sites.

Another possible effect of stimulating the MT enhancer was suggested by our observation that expression of randomly integrated reporter constructs is unstable, as shown above. Clones were removed from G418 (at which point all cells are expressing β-geo) and split into two aliquots; one aliquot was maintained in standard medium and the other supplemented with zinc sulfate (see Fig. 3). At intervals the proportion of cells that continue to express was measured by FACS/Gal. In six of the eight clones expression of β-geo was unstable when G418 selection was removed, in each of these zinc stimulation slowed significantly the rate at which expression was
silenced (Fig. 4B). This rate of silencing is highly variable from clone to clone, and the effect of zinc is also variable. The level of expression is not clearly related to the rate of silencing (cf. clones 19 and 20). In every case in which there is a tendency for expression to be silenced, however, zinc stimulation slows the process significantly. Because stimulation does not increase the level of expression in these clones, stabilization must not require increased expression. Restimulation of silenced cells with zinc does not result in reactivation of expression (not shown).

Discussion

We have studied two distinct effects of enhancers on linked promoters: (1) the level of expression driven by the promoter, and (2) the rate at which expression is silenced. We find that the 5'HS2 enhancer has only a slight effect on the level of expression, and that the MT enhancer has no effect. Both enhancers dramatically slow the rate of silencing, which is highly dependent on the integration site. These results provide strong support for the view that enhancers act on a binary switch in transcriptional control, rather than to regulate the rate of transcription [Weintraub 1988; Moon and Ley 1990; Walters et al. 1995]. Instead of regulating the level of expression, enhancers appear to protect constructs from repression by flanking chromatin; this function is consistent with the role of enhancers in regulating tissue-specific gene expression. Taken together, these results suggest that transcriptional activators may affect directly a binary switch in control of expression that is independent of the mechanism for regulating the rate of transcription.

We find that clones expressing β-geo revert to a silent state at a rate that is different for each clone and presumably a function of the site of integration; this rate is not correlated with the level of expression in the clone. The behavior of these clones is similar to that reported previously for mammary tumor viruses [Feinstein et al. 1982], endogenous avian proviruses [Conklin and Groudine 1986], and some plasmid constructs [Davies et al. 1982], and resembles PEV in that each integration site appears to have a different ability to silence a juxtaposed transcriptional unit [Henikoff 1992; Tartof 1994]. Silencing is associated with methylation and loss of DNase I hypersensitivity at the promoter [D.I.K. Martin, unpubl.], supporting the interpretation that expression in the subclones has ceased rather than simply declined to undetectable levels [however, we do not know whether heterochromatin assembly or methylation is the primary step in silencing]; silenced cells return to an active state at an extremely low rate. Both the 5'HS2 and MT enhancers counteract this silencing effect, and thus function as cis-acting suppressors of PEV.

This result provides direct evidence contrary to the prevailing model of enhancer action, which holds that enhancer-bound activators contact the basal complex and increase the rate of transcriptional initiation [Singer and Berg 1991; Lewin 1994]. In one case we find that MREs placed at a distant site are able to counteract silencing by elements at the integration site; this effect occurs in the absence of any effect on the level of reporter expression. In the other case, removal of the

**Figure 3.** Strategy for study of metal-responsive enhancement. The construct (top) contains the γ-globin promoter driving expression of β-geo, with three copies of the upstream region of the mMT-I promoter downstream of β-geo. This region (−40 to −220) contains the MREs of the promoter [Stuart et al. 1985; Mueller et al. 1988]. Restriction sites used to generate probes for Southern blotting are marked: (R) EcoRI; (X) XhoI; (C) ClaI; (B) BamHI. Probes are denoted by solid lines beneath β-geo. The construct was electroporated into K562 cells and eight single copy integrants were characterized. Basal expression and inducibility were studied by splitting cells maintained in G418 into two aliquots, and adding zinc sulfate to 80 μM to one aliquot. Twenty-four hours later β-gal activity in each aliquot was determined by MUG conversion in cell lysates. Stability of β-geo expression was determined by removing cells from G418 and dividing them into two aliquots. One aliquot was maintained in standard medium, the other in medium with 80 μM zinc sulfate. At intervals FACScan/Gal was performed to assay the proportion of expressing and silenced cells.
5'HS2 enhancer results in only a slight decline in expression, but at many integration sites causes a greatly increased rate of silencing. The stability of expression by the clones is site dependent; this is expected if, as in PEV, chromatin flanking the transcription unit varies in its ability to repress activity in the unit. Thus, our results are consistent with the view that enhancers act to initiate and maintain a chromatin state that permits promoter activity. They are supported by earlier experiments using transient and stable expression assays (Weintraub 1988; Moon and Ley 1990; Walters et al. 1995), as well as a study that found that deletion of an immunoglobulin enhancer resulted in cessation of expression (Grosschedl and Marx 1988). A binary mode of regulation has also been proposed for the rDNA enhancer, which has been shown to regulate the number of active rDNA units, rather than polymerase density (Reeder 1984, 1992).

It should be noted that differences in expression level are seen when a single copy of the same construct is integrated in different sites (Figs. 2A and 4A), suggesting that some characteristic of the chromatin milieu affects either initiation or elongation of transcription, or the proportion of the cell cycle in which transcription is occurring. It may be that enhancers contribute to this milieu and therefore, affect expression, although not in the direct way that is widely supposed; the slight decline in expression when 5'HS2 is deleted supports this view. In this regard, the differences in level that we see (four- to fivefold) among the lines with single integrated plasmids are not large in comparison to those found in lines carrying multiple copies (Walters et al. 1995). Thus, we speculate that the chromatin context of a gene, including the distant cis-acting elements, has a relatively slight influence on a gene's rate of transcription, but a large influence on its ability to remain active.

We propose that many integration sites are sufficiently repressive that activity cannot be established by a promoter in the absence of an enhancer. Although our results are generally in agreement with this hypothesis, we note that in most of the clones we have studied deletion of 5'HS2 does not result in an immediate cessation of

Figure 4. Basal level, inducibility, and stability of β-geo expression in γ/β-geo/MRE clones. (A) Basal and induced expression of β-geo is shown with standard error of the mean. Results shown are means of at least three experiments, and are normalized to the negative control. Induced levels (solid bars) are 24 hr after addition of 80 μM zinc sulphate. There is a fourfold variation of basal expression level between clones (open bars), but no increase is seen with zinc induction. Results of experiments performed with cells maintained free of G418 do not differ significantly from those shown. Eight other clones were analyzed in this way and none showed increased expression upon induction (not shown). (B) Stability of β-geo expression in γ/β-geo/MRE clones expanded with (+) or without (−) 80 μM zinc sulphate in the culture medium. The x-axis in these histograms represents a three-decade log scale of fluorescence. All clones were maintained in G418 until time 0 and then split into induced and uninduced aliquots, therefore at time 0 all were expressing and the two populations were identical. There is a steady accumulation of nonexpressing cells over time. The sequence of histograms labeled C10—represents the progression of silencing in C10 expanded without added zinc sulfate as an example; D denotes day. The paired histograms show FACS/Gal assays of each induced/uninduced pair done after 5 months of growth without G418. Two clones (4 and 19) show no silencing at this point. The other clones show significant, but varying, proportions of silent cells, but in each case zinc stimulation slows or completely prevents silencing.
expression, but merely hastens it (and to highly variable extent). However, inclusion of 5'HS2 in a construct results in a 25-fold increase in the number of expressing clones compared to the γ-globin promoter alone in the colony assay (Moon and Ley 1990; Chung et al. 1993; Walters et al. 1995), implying that there are many more enhancer-permissive than promoter-permissive sites. This apparent disparity (between the effects on initiation and those on maintenance of expression) raises the possibility that in some integration sites an enhancer is necessary to establish expression, but that promoter activity is sufficient to maintain it for many cell divisions over an extended period of time. Establishment of expression may require the disruption of stable structures, whereas maintenance may require only that their formation be prevented. Although it is possible that maintenance of our clones without G418 selection for greatly extended periods would show that deletion of 5'HS2 always results in an eventual silencing of expression, the fact that the deleted subclones can be expanded in G418 strongly implies that maintenance of expression has less stringent requirements than does establishment.

Other instances in which transcriptional activators may counteract directly the repressive effects of chromatin packaging have been noted. In addition to the effects of activators on displacement of nucleosomes (Felsenfeld 1992; Kornberg and Lorch 1992; Paranjape et al. 1994; Wolffe 1994), two instances of this effect have been seen in genetic assays. In Drosophila, genes of the Pc-G establish clonally heritable states of repression in homoecic genes by forming repressive chromatin structures (Zink et al. 1991; Pirrotta and Rastelli 1994; Orlando and Paro 1995). One class of antagonists of Pc-G genes, the trithorax group, encodes transcriptional activators (Farkas et al. 1994); trithorax binds to the polycistron response element identified in the Ubx gene (Chan et al. 1994; Paro 1995). In addition, telomeric silencing of URA3 expression in yeast, a form of PEV, is counteracted by overexpression of Ppr1, a transcriptional activator of URA3 (Renauld et al. 1993; Aparicio and Gottschling 1994).

If one considers that the phenomenon of silencing we observe is likely to have the same basis as PEV and related phenomena such as telomeric position effect, the results of our studies suggest that transcriptional activators may directly inhibit silencing by chromatin structures, and that this effect is independent of the rate of transcription. The function of an enhancer in this model is to antagonize formation of repressive chromatin structures, thus directing tissue-specific expression of some genes, tissue-specific enhancers may ensure the activity of discrete domains in appropriate lineages. We speculate that modulation of transcriptional rate may be a function only of promoter elements, with the basal rate determined by the structure of large areas of flanking chromatin. It is possible that promoter elements also act to overcome repressive chromatin effects. This notion is supported by the observation that when expression of an EBV-encoded protein in human B cells is induced by the binding of a chimeric ZEBRA/VP16 activator to the viral promoter, the level of increase in expression is proportional to the increase in the number of cells in the population actively expressing the protein (Baumann et al. 1993). The fact that many upstream promoter elements are shared with enhancers suggests that these too may antagonize chromatin repression directly rather than, or as well as, regulate rates of transcription.

**Materials and methods**

**Plasmid constructs**

γ/β-geo/HS2–FRT The 1-kb Smal–BglIII fragment of human 5′HS2 of the human β-globin LCR was blunt-ended and ligated to a Hpal site between two FRT elements in the plasmid pGEM-FRT-2. The FRT/5′HS2/FRT cassette was then excised and ligated to a blunt-ended SalI site 3′ to β-geo in the plasmid γ/β-geo (Walters et al. 1995).

pflp/hygromycin The plasmid pOG44, which expresses the flp recombinase under control of the simian cytomegalovirus (CMV) immediate-early promoter/enhancer, was digested with SalI and ligated to a 1.3-kb SalI–XhoI fragment containing the hygromycin phosphotransferase gene driven by the herpes simplex virus (HSV) thymidine kinase promoter. The hygromycin HSV and flp genes were confirmed as residing on opposite strands of the plasmid.

γ/β-geo3MRE A fragment of the mMT-1 promoter from −32 to −220 (Glanville et al. 1981) was amplified by PCR with BglII sites on the ends, digested with BglII, and ligated into the BglII site 3′ of β-geo in γ/β-geo. Recombinants were screened by restriction enzyme digestion to identify those with multiple copies of the fragment; one with three copies (γ/β-geo3MRE) was then sequenced to confirm the presence of the fragment.

**Cell culture and transfection**

Conditions for growth and electroporation of K562 cells were as described (Walters et al. 1995), for induction of MT elements zinc sulfate was added to a concentration of 80 μM 24 hr before the assay. For deletion of 5′HS2, 48 hr after transfection of K562 cells containing a single stably integrated copy of γ/β-geo/HS2–FRT with pflp/hygromycin, cells were plated in soft agar containing Iscove’s medium supplemented with 10% calf serum and 0.4 mg of hygromycin B (Calbiochem) per milliliter. G418 was not included in the selection medium. Clones resistant to hygromycin were isolated in 10–14 days and enhancer removal confirmed by Southern blot (see below).

**Screening for single-copy integrants**

After electroporation of K562 cells with γ/β-geo/HS2–FRT, G418-resistant clones were screened for single copy integrants. Preparation of genomic DNA, restriction digest, gel electrophoresis, membrane transfers, and probe radiolabeling were performed by standard methods (Sambrook et al. 1989). Genomic DNA was digested with EcoRI, which cuts at a single site in the β-geo gene in both γ/β-geo/HS2–FRT and γ/β-geo3MRE. Single integration events were confirmed by using hybridization probes located either upstream (900 bp Clal–BamHI LacZ fragment) or downstream (1.2 kb EcoRI–XhoI β-geo fragment) of the EcoRI site in β-geo. Only those clones that generated a single hybridization signal >4 kb in size (with a different size for each probe) were selected for subsequent analysis. Integrity of the putative single copy integrants was confirmed by digestion of
genomic DNA with EcoRI and XhoI to yield a 2.2 kb junction β-geo/5'HS2 fragment when hybridized to the 1.2-kb EcoRI-XhoI β-geo probe.

flip-mediated deletion of 5'HS2
After transfection with pFlp/hygroycin, hygromycin-resistant γ/β-geo/5'HS2-FRT clones (as described above) were expanded and screened for removal of the 5'HS2 enhancer by flip recombinase. Genomic DNA was digested with EcoRI and XhoI and hybridized to the 1.2-kb EcoRI-XhoI β-geo probe. Clones without an enhancer generated a 1.2-kb hybridization signal that was readily distinguishable from the 2.2-kb signal present in parent controls with an intact enhancer. In many cases, hygromycin-resistant colonies were chimeric for the enhancer deletion. For this reason, single viable cells were sorted on the basis of propidium iodide exclusion from each of the hygromycin-resistant clones; sorting was performed on a Vantage instrument (Becton Dickinson immunocytometry systems). These single cells were expanded and then analyzed a second time by Southern blot to identify clones carrying the enhancer deletion. In addition, parent and derived subclones lacking an enhancer were submitted to Southern blotting at various intervals to confirm that no contamination or rearrangement of the clones had occurred in the course of their analysis.

Determination of β-galactosidase activity
The MUG assay was performed on bulk cellular lysates in 96-well plates on a Dynatech fluorimeter as described previously (Fiering et al. 1990, Walters et al. 1995). Protein content of lysates was determined by the Bradford method, and this content was confirmed to lie in the linear portion of this assay before proceeding with the MUG assay. Fluorescence of each sample was measured in triplicate and mean β-gal activity was determined after correction for protein content of the lysates. In the case of the γ/β-geo/HS2-FRT clones and their deleted subclones, correction for the fraction of cells expressing β-galactosidase (as determined simultaneously by the FACS/Gal assay, see below) was made in each case, therefore a measure of β-gal expression per cell was generated. MUG assays were repeated at least six times at different temporal intervals, and mean activity relative to a reference sample (subclone 19) and standard error of the mean were calculated.

FACS/Gal assays
FACS/Gal analysis was performed as described previously [Nolan et al. 1988; Fiering et al. 1991, Walters et al. 1995]. The γ/β-geo/3MRE clones were assayed at intervals over a period of 4 months after clones were split into standard medium or medium with zinc sulfate. There is no effect of zinc on γ/β-geo clones lacking MT elements. At the start of the comparison of β-gal expression in γ/β-geo/HS2–FRT parent clones and their subclones lacking 5'HS2, populations of β-gal-expressing cells were isolated by sorting on the basis of fluorescence with fluorescein di-β-D-galactopyranoside (FDG). These cells were expanded and analyzed subsequently for β-gal expression by FACS/GAL. Cells were incubated on ice for 120 min in the presence of FDG before analysis, which permitted an adequate interval for detecting any β-gal activity (we did not detect an increase in the proportion of cells counted as positive with incubation intervals of <16 hr). Parent and subclones lacking an enhancer were divided into three aliquots and FACS analysis performed separately at each time interval. Intervals between FACS analysis were typically 7 days, however, for particularly unstable subclones (e.g., clone 25), FACS analysis was performed daily. Cells were scored as β-gal expressing if their fluorescence level exceeded the level of 99.9% of negative control K562 cells; the proportion of β-gal-expressing cells for each of the parents and subclones was tabulated for each of the time intervals. At each interval the MUG assay was performed on the same day as the FACS/Gal assay, permitting calculation of an estimate of β-gal expression per cell, as described above.

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