Experimental Cell Research **274,** 1–8 (2002)
doi:10.1006/excr.2001.5443, available online at http://www.idealibrary.com on IDEAL®

Evidence for Repeat-Induced Gene Silencing in Cultured Mammalian Cells: Inactivation of Tandem Repeats of Transfected Genes

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Foreign DNA can be readily integrated into the genomes of mammalian embryonic cells by retroviral infection, DNA microinjection, and transfection protocols. However, the transgenic DNA is frequently not expressed or is expressed at levels far below expectation. In a number of organisms such as yeast, plants, *Drosophila*, and nematodes, silencing of transfected genes is triggered by the interaction between adjacent or dispersed copies of genes of identical sequence. We set out to determine whether a mechanism similar to repeat-induced gene silencing (RIGS) is responsible for the silencing of transgenes in murine embryonal carcinoma stem cells. We compared the expression of identical reporter gene constructs in cells carrying single or multiple copies and found that the level of expression per integrated copy was more than 10-fold higher in single-copy integrants. In cells carrying tandem copies of the transgene, many copies were methylated and clones frequently failed to express both copies of near-identical integrated alleles. Addition of extra copies of the reporter gene coding sequence reduced the level of expression from the same reporter driven by a eukaryotic promoter. We also found that inhibitors of histone deacetylase such as trichostatin A forestall the silencing of multicopy transgenes, suggesting that chromatin mediates the silencing of transfected genes. This evidence is consistent with the idea that RIGS does occur in mammalian embryonic stem cells although silencing of single-copy transgenes also occurs, suggesting that RIGS is only one of the mechanisms responsible for triggering transgene silencing.

Key Words: **transfection; embryonal carcinoma; cell culture.**

INTRODUCTION

The expression of transgenes in mammalian cells or organisms is often disappointing. In many instances, the genes are not expressed at all or are expressed at levels far lower than anticipated. This problem seems particularly evident when multiple copies of transgenes are integrated and the level of expression does not reflect the number of transgene copies. In instances in which expression of the transgene can be assessed in individual cells, populations of genetically identical cells frequently express the transgene in a variegated or mosaic fashion [1]. Certain DNA sequences (called locus control regions) added to the transgene have been shown to improve expression levels, perhaps by creating a chromatin environment conducive to maintaining active expression [2]. The nature of the signals that silence the transgenes is not known although it is often attributed to "site of integration" where the transgene or cluster of transgenes is thought to come under the influence of presumed long-range chromatin-modifying processes.

In lower eukaryotes and in plants, transgene silencing has been studied extensively. The absence of transgene expression can be due to transcriptional or posttranscriptional events. Posttranscriptional gene silencing is now known to be caused by double-stranded RNA functioning through a sequence-specific RNA degradation mechanism called RNA interference [3–5]. Silencing that results from failure to transcribe the transgene is also common, and this mode of silencing can be triggered by the presence of multiple copies of the transgene. This so-called repeat-induced gene silencing (RIGS) has been documented in fungi [6], plants [7], *Drosophila* [8], and nemotodes [9] and appears to be triggered by direct or inverted repeats of gene-sized DNA regions of identical sequence [10]. The efficiency of silencing by RIGS is modulated by proximity of the transgene cluster to large blocks of heterochromatin [8, 11] and is thought to be induced by interaction between the tandem copies and even distal copies of the genes present in multiple copies. Evidence that RIGS may also occur in mammals has been reported [12] in a study showing that the expression of a transgene was enhanced in two lines of transgenic animals following Cre-mediated reduction in transgene copy number.

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0014-4827/02 $35.00

We have been studying the expression of genes transfected into embryonal carcinoma stem cells. We found that expression of transgenes integrated into the genome is mosaic [13] and invariably very unstable in these cells [14]. The mosaic expression results from the irreversible inactivation of transgenes that occurs during propagation of the transformed cells [15]. Silencing occurs at the transcriptional level as demonstrated by nuclear run-on experiments [15]. We found that the efficiency with which the transfected genes were expressed in stably transformed cells could be enhanced by cotransforming cells with the murine *Pgk-1* gene or a portion of this gene that contained both the promoter and at least several introns and exons [16, 17]. We interpreted this enhanced expression to indicate that the *Pgk-1* gene functions as a boundary element or insulator between the repeated copies of the unselected transgenes that are normally silenced by a process in mammalian cells analogous to RIGS. We set out here to provide more direct evidence for RIGS in mammalian cells by creating transgenes carrying single or multiple copies of a reporter gene. The results are consistent with the idea that RIGS does occur in mammalian cells. However, inactivation of single-copy transgenes does occur in our cells, suggesting that other mechanisms for transgene silencing also exist.

MATERIALS AND METHODS

Cell culture. The studies reported here were carried out with the P19 line of murine embryonal carcinoma stem cells [18] cultured under conditions described [19]. These cells were transfected by plating 10^6 cells in a 60-mm culture dish and using 10 μg of circular plasmid DNAs with a modified calcium phosphate coprecipitation method [20]. Alternatively, cells were electroporated in a suspension of 5×10^6 cells along with 25 μg of linearized plasmid DNA in a Bio-Rad GenePulser plus Capacitance Extender set at 500 μF and 300 V. Following transfection or electroporation, cultures were incubated for 48 h and then replated into 150-mm culture dishes at 2×10^6 cells per dish in medium supplemented with puromycin (2 μg/ml) or G418 (400 μg/ml), depending on the nature of the selectable gene used to transform the cells. Medium was changed after 4 days and cultures were harvested at 8 days when typically 1000–2000 colonies were present.

Spectrophotometric assays for β-galactosidase were carried out as described [15], and *in situ* staining for β-galactosidase was carried out with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as described [15].

Molecular genetics. DNA was isolated from transfected cells [21] and dissolved in TE buffer. This DNA was digested with restriction enzymes at 10 U/μg before being run on 1% agarose gels in TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) [22]. The DNA was then transferred to Hybond-N membranes (Amersham) and treated with ultraviolet light at 110 mJ using a GS Gene Linker UV chamber (Bio-Rad). These blots were probed with isolated DNA fragments radiolabeled with [^{32}P]-dCTP by random priming [22].

The eukaryotic expression vectors used in this study comprised coding sequences from the *Escherichia coli lacZ* gene, the puromycin-resistance gene (*puro*) [23], the *lacZ-neo* fusion gene [24], or the enhanced green fluorescent protein (EGFP) under the control of the murine *Pgk-1* promoter and polyadenylation/transcription termination sequences [25, 26]. In some experiments, the plasmids carried one expression vector, while in others, two expression cassettes were present in the same plasmid.

RESULTS

Multicopy Transgenes Are Expressed Inefficiently

To determine whether RIGS occurs in mammalian cells, we set out to examine the expression of reporter genes in P19 cells when the reporter genes are present in single or multiple copies in the genome of transformed cells. For these experiments, we used the TM20 construct, which carries one eukaryotic gene comprising the *Pgk-1* regulatory sequences [25, 26] driving the *lacZ-neo* gene, which encodes a fusion protein formed between the *E. coli lacZ* gene and the neomycin-resistance gene [24]. This construct was linearized and electroporated into P19 cells under conditions under which only single copies of the plasmid are normally integrated into the genome. The same plasmid was transfected by the calcium phosphate method under conditions under which multiple copies of plasmids are normally co-integrated into the genomes of stably transformed cells [19, 27]. Cells carrying and expressing the plasmid-derived *lacZ-neo* gene were selected in G418, the colonies were pooled, and β-galactosidase was measured in extracts from these cell populations. DNA was isolated from the same cell populations and this DNA was used in slot blots subsequently probed with *lacZ* sequences to determine the average number of copies of the *lacZ-neo* transgene integrated per transformed cell.

The levels of expression of β-galactosidase expressed from the *lacZ-neo* gene were slightly higher in electroporated than in transfected cells. However, the number of transgene sequences integrated into the genomes of selected cells was much higher in cells that had been transfected using the calcium phosphate method (Fig. 1A). Thus, the level of β-galactosidase activity per integrated *lacZ-neo* gene was at least 10-fold higher in cells that were electroporated.

Only cells that express at least one copy of the *lacZ-neo* gene would be represented in the analysis shown in Fig. 1A because colonies were selected for G418 resistance. We repeated this experiment with a plasmid, pKJ35, that carries two different eukaryotic genes, one encoding puromycin resistance and one encoding β-galactosidase. Each gene has its own promoter and polyadenylation signals derived from the murine *Pgk-1* gene [25, 26] (Fig. 1B). Transfected and electroporated cells were selected for puromycin resistance and analyzed for expression of β-galactosidase from the nonselected *lacZ* gene. The results, shown in Fig. 1B, indicate that the *lacZ* gene is expressed efficiently in both transfected and electroporated cells, that the number of copies of the *lacZ* gene is much higher in transfected

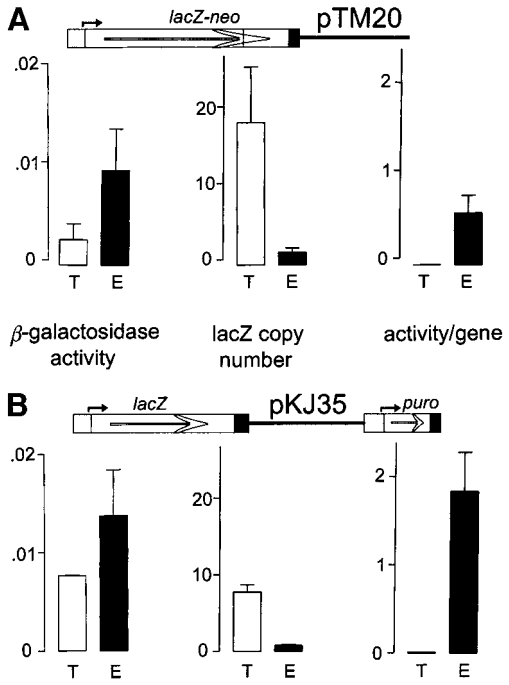

FIG. 1. Inefficient expression from multicopy transgenes following calcium phosphate-mediated transfection. (A) P19 cells were transfected (T) or electroporated (E) with the plasmid pTM20, a plasmid carrying one gene consisting of the *Pgk-1*-derived promoter (gray box) [25] and *Pgk-1* polyadenylation and transcription termination signals (black box) [26] flanking the *lacZ-neo* coding region [24]. *LacZ-neo* (also called βgeo) consists of a fusion between the coding regions of *lacZ* and the neomycin-resistance gene. The solid line represents the plasmid vector backbone. Cells were cultured in the presence of G418, the colonies formed after 7 days were pooled, and the β-galactosidase activity was measured in the populations of pooled cells. The DNA from these same cells was isolated and slot blots containing this DNA were probed with the *lacZ* gene to estimate the average number of transgene copies per cell. The average β-galactosidase activity per integrated *lacZ* gene was calculated and is shown on the right. (B) P19 cells were transfected or electroporated with the plasmid pKJ35, which carries two recombinant genes as shown in the diagram at the top. Both the *lacZ* coding region and the *puro* coding region are regulated by the murine *Pgk-1* promoter [25] and 3′ end. Cells were cultured in the presence of puromycin and the colonies formed were pooled and processed as for A. The results shown were derived from three or four independent experiments in which the number of colonies pooled for each determination varied from 270 to 2110 for electroporations and 2600 to 6000 for transfections [26]. For transfections, circular plasmids were used to create a fine precipitate in BES-buffered calcium phosphate [19] while for electroporations, the same plasmids were linearized at a unique restriction site before being mixed with cells for electroporation.

than in electroporated cells, and that the activity per integrated *lacZ* gene is much higher in electroporated than in transfected cells.

The *lacZ* sequence is rich in the CpG dinucleotide. In previous experiments we showed that silent *lacZ* genes were heavily methylated at all 15 CpG sites assessed [28]. The methylation-sensitive enzyme *Pau*I has one restriction site in the *lacZ* gene and we used this enzyme to assess the level of methylation of *lacZ* sequences in cells carrying transfected or electroporated copies of pKJ35 or pTM20. DNA from pooled colonies was first digested with *PvuII* to release a 2.5-kb *lacZ* fragment and aliquots from the same samples were subsequently digested with *Pau*I. Control experiments with genomic DNA mixed with plasmid DNA indicated that *Pau*I digestion went to completion under the digestion conditions used. In *lacZ* sequences that are methylated at the *Pau*I site, the 2.5-kb fragment remains undigested, while in unmethylated *lacZ* copies, the 2.5-kb *PvuII* fragment is digested into two fragments of 1.4 and 1.1 kb. In electroporated samples, the vast majority of *lacZ* copies were unmethylated (Fig. 2, lanes 4 and 8), whereas in transfected cells carrying multiple *lacZ* copies, approximately half appeared to be methylated (Fig. 2, lanes 2 and 6).

One interpretation of the result of Fig. 1 and 2 is that many of the *lacZ* or *lacZ-neo* genes in cells carrying multiple copies of the plasmids are not transcribed. To test this idea directly, we modified the *lacZ-neo* gene to create two nearly identical alleles, a and b, that can be distinguished by a PCR-based procedure (see Fig. 3A). Plasmids carrying the two *lacZ-neo* genes were mixed in equal amounts and cotransfected into P19 cells under conditions under which an average of 10–20 copies are integrated per cell. We selected cells that expressed the *lacZ-neo* gene product by growing them in G418 and analyzed 10 clones of transformed cells to determine whether each expressed one or both alleles. The results reported in Fig. 3B show that 8 of the 10 clones analyzed carried both alleles and that 5 of these 8

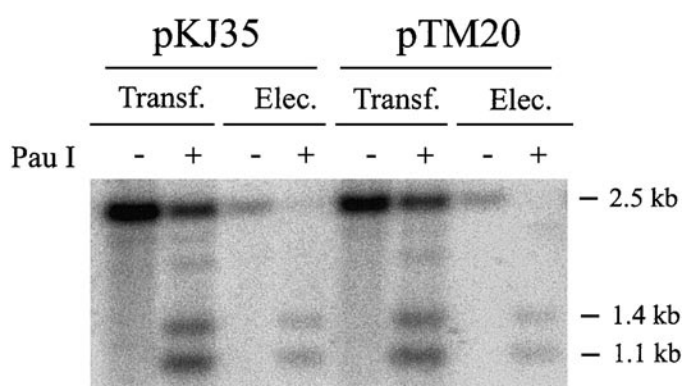

FIG. 2. *De novo* methylation of the *lacZ* sequence in multicopy transformants. P19 cells that had been transfected or electroporated with the constructs described in Fig. 1 were selected in puromycin. At 7 days following transfection, cells were pooled and DNA was isolated. This DNA was digested with *PvuII* to release a 2.5-kb fragment from within the *lacZ* coding region. Every second lane was also digested as well with *Pau*I, a methylation-sensitive enzyme that cuts the 2.5-kb *lacZ* fragment into 1.1- and 1.4-kb pieces. In these and other experiments, we found that about half of the *lacZ* sequences were methylated in transfected cells, whereas almost none of the *lacZ* sequences were methylated in cells that were electroporated.

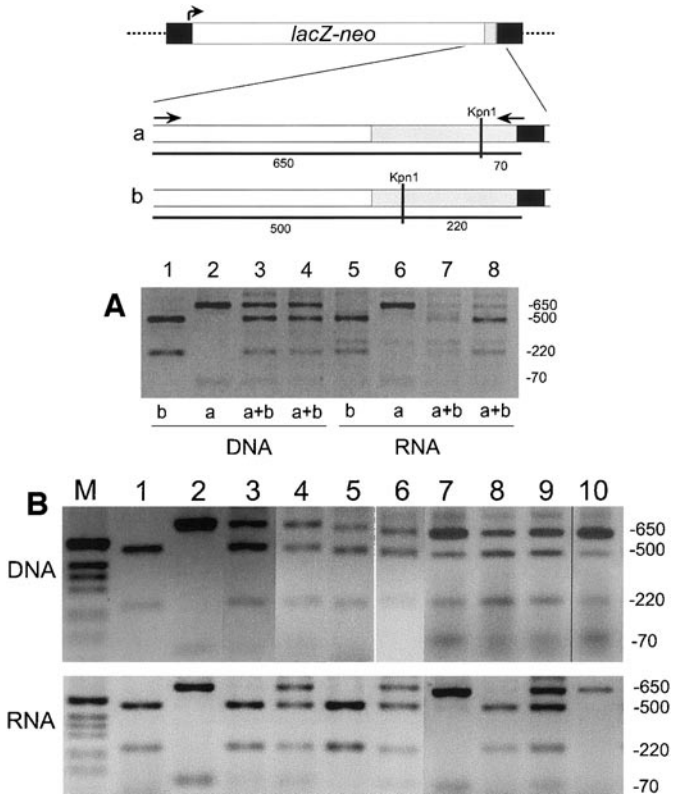

FIG. 3. Clones of stably transfected cells express from only some of the integrated genes. The constructs shown at the top show the modifications of the *lacZ-neo* gene that were made to create the *lacZ-neo*^{a} and *lacZ-neo*^{b} alleles. A 296-bp *Nhe*I–*Spe*I fragment (shaded box) from the mouse *sir2* gene was inserted in both orientations into the unique *Xba*I site in the 3′UTR of the *lacZ-neo* gene. This fragment carries a *Kpn*I site 70 bp from its end. Oligonucleotide primers (arrows above the a allele) from the *neo* gene and from the *Pgk-1* 3′UTR were used to amplify 720-bp fragments from both genes and *Kpn*I digestion of the amplified product predicts fragments of 650+70 bp from the *lacZ-neo*^{a} allele and 500+220 bp from the *lacZ-neo*^{b} allele. The two plasmids were used individually or mixed in equal amounts to transfect P19 cells under conditions (Fig. 1) under which 10–20 plasmid copies per genome are integrated. Following transfection, cells were selected in 400 μg/ml G418 for colony formation. In (A), colonies of selected cells were pooled and DNA and RNA isolated from each culture. In lanes 1–4, the DNA from these cultures was used as the template for amplification of the 720-bp fragment that was digested with *Kpn*I and analyzed on agarose gels. In lanes 5–8, RNA from the same samples was reverse transcribed (primed with oligo(dT)), amplified, and digested with *Kpn*I. Lanes 1 and 5 are from cultures transfected with *lacZ-neo*^{b}-alone, lanes 2 and 6 are from cultures transfected with *lacZ-neo*^{a}-alone, and lanes 3, 4, 7, and 8 are from cultures transfected with two separate mixtures of *lacZ-neo*^{a}- and *lacZ-neo*^{b}. The fragment sizes are shown on the right and the alleles present indicated at the bottom. (B) The PCR and RT-PCR results from DNA (above) and RNA (below) isolated from 10 clones of cells expanded from cultures transfected with mixtures of both plasmids. Clones 1 and 2 apparently incorporated only the *lacZ-neo*^{b} and *lacZ-neo*^{a} alleles, respectively, while clones 3–10 had incorporated both alleles. However, clones 3, 5, 7, 8, and 10 expressed only one of these two alleles.

clones expressed only one of the two *lacZ-neo* alleles present in their genome.

Extra Copies of the lacZ Sequence Reduce Expression from Pgk-lacZ

Cells transfected with the calcium phosphate method have widely varying numbers of copies of transgenes [15]. To more carefully control the copy number and to determine whether extra copies of the *lacZ* sequence could reduce expression from *Pgk-lacZ*, we modified pKJ35 to add extra copies of the *lacZ* coding region. These new constructs, pKJ163 and pKJ167, carried one and two copies, respectively, of *lacZ* upstream of *Pgk-lacZ* oriented in the same direction as the *Pgk-lacZ* sequence (Fig. 4). Following electroporation into P19 cells and selection in puromycin, cells were harvested and the levels of β-galactosidase assessed. The addition of one and two extra copies of the *lacZ* sequence resulted in decreased expression from the *Pgk-lacZ* reporter gene.

As a control, we created similar constructs carrying, instead of *lacZ*, a 3-kb sequence from the body of the *Pgk-1* gene. In constructs pKJ164 and pKJ170, this 3-kb region was present in one or two copies in its positive orientation upstream of *Pgk-lacZ*. A single copy of this sequence had no effect on expression (KJ164), while two copies (KJ170) reduced expression.

Although the magnitude of the effect of *lacZ* repeats on *Pgk-lacZ* expression was not large, there was significantly reduced expression in constructs carrying two extra copies of *lacZ*. We interpret this result to be consistent with the notion that direct repeats of DNA sequences increase inactivation of neighboring reporter genes.

If the extra copies of *lacZ* were triggering gene inactivation, one might expect that the proportion of cells carrying silenced *Pgk-lacZ* would increase with time in culture. We picked a number of clones transformed with each plasmid, cultured them continuously in the absence of puromycin, and assessed the proportion of cells in each culture that was X-gal positive. Figure 5 shows the results from one experiment in which clones of cells transformed with each of the plasmids were cultured in the absence of selective pressure. Although there was considerable variability, cells transformed with each of the plasmids showed the same downward trend in the proportion of cells that retained expression of β-galactosidase. Somewhat surprisingly, even in cells containing a single copy of the *Pgk-lacZ* sequence (pKJ35) there was progressive loss of expression with time in culture. Similar trends toward progressive inactivation of genes transfected into cultured cells have been documented by others [29, 30], indicating that progressive silencing of transgenes is a very common if not universal phenomenon.

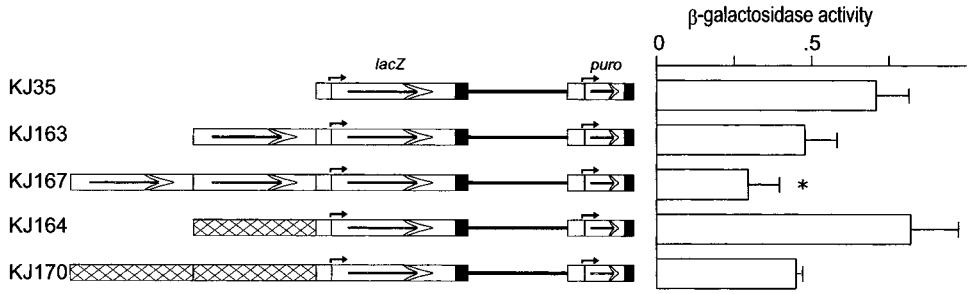

FIG. 4. Copies of the *lacZ* coding region decrease expression from a linked *Pgk-lacZ* gene. The constructs shown on the left were linearized and electroporated into P19 cells. Cultures were then grown in the presence of puromycin and β-galactosidase activity was measured in pooled populations of drug-resistant cells. The gray boxes represent the *Pgk-1* promoter used to drive both the *lacZ* and the *puro* genes. Black boxes represent the polyadenylation and transcription termination signals from the 3′ end of *Pgk-1*. The white boxes with long arrows indicate the *lacZ* coding region. This *lacZ* region without a promoter is duplicated once and twice in constructs KJ163 and KJ167. In constructs KJ164 and KJ170, a 3-kb fragment (represented as cross-hatched boxes) from the body of the *Pgk-1* gene was inserted once and twice, respectively, in the positive orientation as a control for plasmid size. This 3-kb region is a *Nae*I fragment spanning exons 3 to 5 of the murine *Pgk-1* gene [36]. The dark line represents the 3-kb vector sequence carrying the bacterial *amp* and *ori* regions. The experiment was carried out nine times for constructs KJ35, KJ163, and KJ164, six times for KJ167, and twice for KJ170. Activities of β-galactosidase are reported as change in optical density at 420 nm/30 min/200 μg protein. The asterisk next to the activity measurement from KJ167 indicates that this value is significantly different from that of KJ35 ($P = 0.02$ in a two-tailed t test).

That cells electroporated with pKJ35 have variable expression of the *lacZ* gene was evident in the colonies formed following puromycin selection (Fig. 6). Some colonies failed to stain with X-gal, indicating that they had undetectably low levels of β-galactosidase. Other colonies consisted of cells that stained uniformly with X-gal. However, most colonies were mosaics of cells that were X-gal positive and X-gal negative. We previously noted that many of the colonies formed following transfection with the pKJ35 plasmid were comprised of cells that were heterogeneous with respect to β-galactosidase expression [14, 15]. Thus, silencing of the *lacZ* gene does not require that the silenced gene be present in multiple copies.

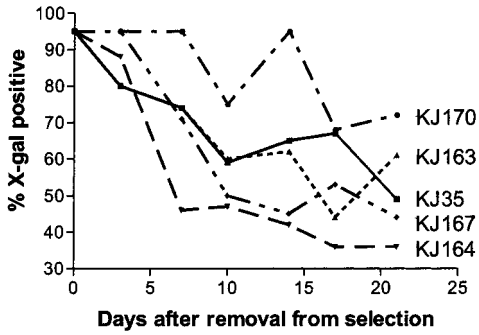

FIG. 5. Loss of expression from *Pgk-lacZ* in stably transformed clones. P19 cells were electroporated with the constructs shown in Fig. 4 and colonies selected in puromycin. At day 7, clones of cells transformed by each plasmid were isolated and expanded. One clone transformed with each plasmid was selected based on that clone having more than 95% of the cells expressing β-galactosidase. These clones were cultured in the absence of puromycin and the proportion of cells that stained with X-gal was assessed at intervals. In each clone the proportion of cells that was X-gal positive decreased with time.

Inhibitor of Histone Deacetylase Enhances Expression from Multicopy Transfection

The results discussed above suggest that repeats of the *lacZ* or *lacZ-neo* gene can be silenced during or following integration into the genome. Silent chromatin is characterized by a low level of acetylation of core histones. We previously showed that trichostatin A (TSA) and butyrate, two inhibitors of histone deacetylase, can enhance the activity of transfected genes [17].

We carried out electroporation experiments in the presence and absence of TSA and found that the drug had no effect on either the number of colonies formed or the level of expression of β-galactosidase from either *Pgk-lacZ* or *Pgk-lacZ-neo* (data not shown). TSA did, however, significantly enhance the expression of *Pgk-lacZ* when transfected into cells by calcium phosphate

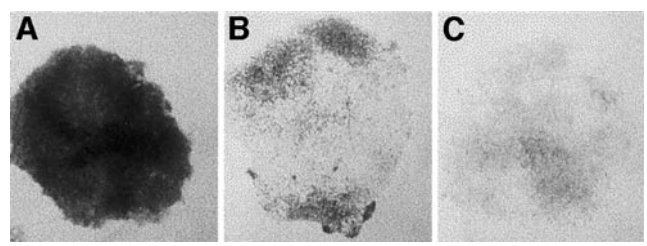

FIG. 6. Colonies stably transformed by electroporation with pKJ35 are heterogeneous in their expression of β-galactosidase. P19 cells that had been electroporated with pKJ35 were cultured in the presence of puromycin and colonies of drug-resistant cells formed after 7 days were fixed and stained with X-gal. Representative colony types are shown. (A) A colony in which all cells are stained with X-gal, (B) a colony with a mosaic staining pattern, and (C) a colony that failed to stain. More than 80% of all colonies had the mosaic staining pattern with variable proportions of X-gal-stained and unstained cells.

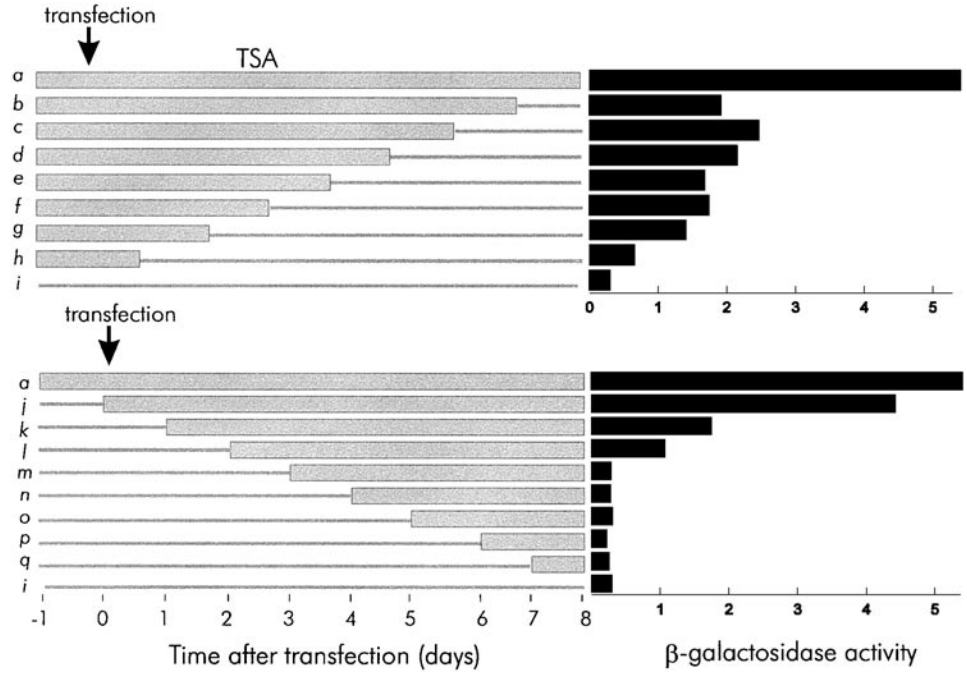

FIG. 7. Histone deacetylation is required for reduced expression from multicopy transgenes. P19 cells were cotransfected at time 0 with three plasmids, one carrying *Pgk-lacZ*, one carrying *Pgk-puro*, and one carrying a region of the 5′ end of the *Pgk-1* gene lacking the promoter [17]. At 24 h after transfection the cultures were plated into medium containing puromycin and incubation continued for another 7 days. Following colony formation, the cultures were harvested and β-galactosidase activity was measured in the pooled population of puromycin-resistant colonies. In culture *a*, trichostatin A (TSA, 20 nM) was present continuously in the culture from the day before transfection as indicated by the thick gray lines, while in culture *i* there was no TSA exposure, as indicated by a thin gray line. In all other cultures the TSA was removed (cultures *b* through *h*) or added (cultures *j* through *q*) during the culture period as indicated on the left. The activity of β-galactosidase in each culture is indicated on the right.

coprecipitation (Fig. 7). In cells cultured continuously in the presence of TSA, the level of β-galactosidase expression was at least 10-fold higher than in cells cultured in the absence of drug. The TSA had to remain in the culture for enhanced expression. When TSA was removed at intervals following transfection (Fig. 7, top), the level of expression dropped. And TSA did not enhance expression of transfected genes if it was added more than 2 days after transfection (Fig. 7, bottom).

The kinetics with which TSA addition becomes ineffective suggests that the inactivation of transfected genes occurs within the first 1–2 days after transfection, i.e., at or shortly after integration into the cellular genome. However, silencing does not occur exclusively at early times following integration because removal of TSA results in reduced levels of expression. This suggests that inactivation of the transfected genes can be forestalled with inhibitors of histone deacetylases but a characteristic of the transgenes remains that marks the transgene cluster for subsequent inactivation when TSA is removed. TSA addition following transfection has little effect, suggesting that the drug does not reactivate expression of transfected genes after these genes have been silenced, a result consistent with a number of other observations in mammalian cells carrying silent genes [31].

DISCUSSION

The inactivation of trangenes is a serious limitation to gene therapy and to experimental studies requiring stable transgene expression. The silencing of transgenes is associated with DNA methylation but the causal relationship between gene silencing and the hypermethylation of its DNA remains controversial and a number of investigations suggest that events other than DNA methylation trigger gene silencing. The major conclusion from our work is that extra copies of a gene sequence reduce expression from a reporter gene, a conclusion consistent with the idea that tandem repeats of gene sequences trigger silencing in mammalian stem cells. A previous report is also consistent with RIGS occurring in mice carrying a *lacZ* transgene [12].

In previous experiments that investigated the cluster of transfected genes in a clone of P19 cells we found that all copies except one were heavily methylated and that the single unmethylated copy was unmethylated

over the entire *lacZ* coding region [28]. This strongly suggested that only this one unmethylated copy of the transgenes was active and that all others were silent. The β-galactosidase activities from cultures transfected and electroporated with the same constructs were comparable (Fig. 1), consistent with the idea that only one copy of the transgene was active in each transformed clone. Some of the clones of cells carrying two alleles of *lacZ-neo* failed to express both alleles, a result one would expect if many of the transfected genes were silenced shortly after integration into the host cell genome. In fungi, plants, and *Drosophila*, RIGS results in all copies becoming inactivated [10]. In our experiments, we require expression from at least one copy because we apply selective pressure for drug resistance. Cells in which all copies were silenced would not be selected in our experiments.

In *Drosophila* and *Caenorhabditis elegans*, silencing of multicopy transgenes is dependent on the presence of the product of the *Enhancer of Zest* or *mes-2* [9] gene, respectively. The murine genome contains two homologues of these genes called *enx-1* and *enx-2*. Both are expressed in P19 cells; *enx-1* is expressed at a particularly high level (data not shown). Whether one or both of the proteins encoded by these genes are required for mammalian RIGS remains to be determined.

The addition of extra copies of the *lacZ* coding region reduced the expression from *Pgk-lacZ* in stable transformants, a result consistent with the idea that RIGS occurs in mammalian stem cells. However, even in those cells transformed with a single copy of the plasmid pKJ35, many of the transformed clones expressed *lacZ* in a mosaic fashion and, in clones selected for high level of expression, the expression from *lacZ* declined with time in culture, indicating that repeated copies of the gene are not the only means by which cells trigger gene silencing. KJ35 does carry two copies of the *Pgk-1* promoter and polyadenylation sequences controlling expression of both the *lacZ* and the *puro* genes. However, we have found that similar silencing occurs in cells electroporated with *lacZ-neo* in which there are no repeated sequences. In the absence of repeats, some other characteristic must identify the single-copy transgene as a substrate for gene silencing. Since cellular genes are normally not subject to silencing, the transgenes must be marked in some way that targets them for silencing even many generations after the transgene has integrated into the genome. Our experiments use transgenes with prokaryotic codon usage and there has been some evidence that this may trigger silencing in transgenic mice [32]. Consistent with this idea, the EGFP reporter gene, which has been engineered to have mammalian codon usage, is more efficiently maintained in the active state following electroporation into EC and ES cells (M. Lemieux *et al.*, unpublished). The reporter genes we use as well as the vector backbone are also rich in the CpG dinucleotide. The relative density of CpG is low in most regions of the mammalian genome and prokaryotic DNAs, which have higher CpG density, are recognized by both membrane receptor [33] and intracellular [34] proteins. The intracellular mechanism involves DNA-PK and this molecule could interact with the CpG-rich transgenes to trigger silencing. Finally, our transgenes have relatively long open reading frames but contain no introns. In previous experiments, we [17] and others [35] found that transgenes carrying introns were more actively expressed following stable integration into the genome, suggesting that there might be a relationship between the maintanence of active chromatin structure and the local presence of the splicing apparatus.

The reduced expression of transfected genes that we attribute to silencing can be partially forestalled with an inhibitor of histone deacetylases (TSA). The kinetics with which TSA affects expression from transfected genes suggests a mechanism by which silencing occurs. TSA was most effective in enhancing expression if the drug was present during transfection, and the effect of the drug was reduced if added to cultures after transfection. TSA was unable to enhance expression if added more than 3 days following transfection. This suggests that most of the silencing occurs very shortly after integration of genes into the genome. In experiments in which the TSA is removed from the cultures following transfection, it seemed clear that the drug had to remain present in the culture and its removal even several days after transfection led to reduced expression from the reporter genes. This result seems to indicate that the tandem array of gene copies in the transgene cluster remains susceptible to silencing for many days after transfection. The simplest interpretation of these results is that the tandem copies of the transgene form a complex that is very susceptible to silencing, that TSA inhibits the silencing process, but that once silencing has occurred, the inhibition of histone deacetylases is insufficient to reverse the mechanisms responsible for maintaining the silent state.

This work was supported by grants from the National Cancer Institute of Canada and the Canadian Institutes of Health Research.

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Received September 3, 2001
Revised version received November 5, 2001
Published online January 22, 2002