Articles

Transcriptional Properties of Genomic Transgene Integration Sites Marked by Electroporation or Retroviral Infection†

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ABSTRACT: As a possible consequence of their survival strategy, proviruses are predominantly found in transcription-promoting genomic sites. For certain applications, these findings have led to the preferential use of retroviral vectors for the stable integration of transgenes. This study demonstrates that transcription levels of single-copy proviruses, which have been established either by infection or by single-copy transfection (electroporation), are rather comparable. Therefore, electroporation is suggested as an alternative gene transfer route in cases where the use of infectious retroviral vehicles is to be avoided due to safety considerations. A difference between clones derived from these two gene transfer routes concerns the inactivation pattern which, for electroporated clones, is an exclusive property of the low expressers. This difference may be due to the nature of the illegitimate recombination event which is thought to be less invasive if catalyzed by the retroviral integrase. Substantial differences between infection and Ca phosphate-mediated transfection that have been reported earlier are explained by the respective transfection parameters.

A major problem in molecular biology, bioengineering, and gene therapy is the variable expression of transgenes after chromosomal integration. One solution to this problem is sought in the use of retroviral vectors which exploit the ability of the retroviral integration machinery to introduce a single intact copy into a subclass of apparently favorable genomic sites.

Retroviral integration is one of the best defined models for nonhomologous recombination processes due to its integrase-catalyzed, precise insertion mechanism (*1*). This model has therefore permitted the recovery of authentic sets of flanking sequences which seem to have markers in common with highly expressed endogenous genes. Among these are the apposition of a DNAse I hypersensitive site (*2*, *3*), the presence of CpG islands (*4*), and adjacent stretches of bent DNA (*1*) which are structured in a way that discourages nucleosomal assembly (*5*). The precise nature of the integration process has made possible a reconstruction of the original sites prior to integration and revealed a number of structural features, among these their affinity for the nuclear matrix (*5*, *6*).

One rationale for the preferred use of retroviral vectors is an early report by Hwang and Gilboa (*7*), who found that a gene introduced by viral infection is expressed at 10−50-fold higher levels than a copy of the same DNA which has entered the cell by Ca phosphate transfection. These findings appeared to be in contrast to observations from this laboratory regarding the efficiency of infection and electroporation (*5*). Unlike other transfection techniques, electroporation can be optimized to introduce a single copy or only a few copies of a gene, overcoming the now established phenomenon of copy number-dependent gene silencing [also called "cosuppression" (*8*)]. Under these conditions, we noted a rather minor difference in initial expression levels for genes transferred by either technique (*5*, *6*).

These observations together with concerns regarding the biological safety of retroviral systems (*9*) and problems with host cell permissiveness to retrovirus infection (*10*) motivated the present search for alternatives. Here we correlate the immediate and long-term expression characteristics, exclusively of single-copy clones, which contain the transgene in a similar constitution whether it has entered the cell by infection or electroporation. We will demonstrate that for many purposes single-copy electroporation will be a true alternative since it leads to one class of clones with expression parameters closely resembling those of a control group that has been established by infection. Our studies are performed on a baby hamster kidney cell line which has found wide application for the production of pharmaceutical proteins, including a number of blood clotting factors. This cell line (BHK-A) can be infected by common MPSV[1]

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[1] Abbreviations: β-geo, fusion gene from the β-galactosidase reporter and the neomycin resistance gene; 5-azaC, 5-azacytidine; FACS, fluorescence-activated cell sorting; FDG, fluoresceine di-β-galactoside; IRES, internal ribosomal entry site; LTR, long terminal repeat; MPSV, murine proliferative sarkoma virus; MUG, 4-methylumbelliferyl-D-galactoside; TSA, trichostatin A.

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Table 1: Optimized Electroporation Conditions for Single-Copy Integrations[a]

condition	amount of electroporated DNA (μg per 10^6 cells)	parameters (V/μF/ms)	one copy (%)[b]	two copies (%), tt;hh;ht (%)[c]	more than two copies (%), tt;hh;ht (%)[c]
control	0	360/500/7.4	0	0	0
1	4.5	360/500/7.5	6/46[a] (**13**)	12/46 (26), 2;4;0	28/46 (61)
2	2.75	360/1100/17.4	13/47 (**28**)	8/47 (17)	26/47 (55), 2;4;4
3	2.75	540/500/7.9	2/7 (**29**)	3/7 (42)	2/7 (29)
4	2.75	410/500/7.5	17/46 (**37**)	15/46 (33), 2;0;0	14/46 (30), 2;0;4

[a] When one begins from a standard protocol (condition 1), the DNA concentration was lowered and the capacity and voltage of the gene pulser were varied. Single-copy clones from protocols 1−4 together formed the basis for the stability data depicted in in Figures 3 and 4. [b] Number of clones carrying the copy number/the number of clones examined. [c] tt, tail-to-tail; hh, head-to-head; ht, head-to-tail.

(murine proliferative sarcoma virus)-derived retroviruses, although these do not belong to the normal host range (*11*) and the cell line is amenable to a variety of transfection techniques.

MATERIALS AND METHODS

Plasmids. The retroviral expression plasmid pM5βgeo is a derivative of pM5SEPA (*5*). It comprises the retroviral sequences necessary for an efficient transcription and packaging, derived from MPSV. For its construction, the coding sequences were excised from pM5SEPA by *Hin*dIII−*Cla*I cuts and replaced by the 3.9 kb β-geo gene from the plasmid pPGKβgeo-Flp-HS2 (kindly provided by D. Martin, Seattle, WA) which was cut with *Hin*dIII−*Xba*I. For cloning via the blunt end, the *Cla*I and *Xba*I sites had to be filled in by Klenow polymerase.

Cell Culture, Transfection, and Electroporation. Baby hamster kidney cells, strain BHK-A (*11*), a subclone of BHK-21 (ATCC CCL-10), and ψ-2 packaging cells (*12*) were cultured in DME medium containing 10% fetal calf serum, 20 mM glutamine, 60 μg/mL penicillin, and 100 μg/mL streptomycin and passaged by splitting 1:40 at the time of confluence, i.e., after 4 days. This corresponds to five to six population doublings per passage. Transfection routines were as described previously (*13*) using calcium phosphate coprecipitation of circular plasmids. For electroporation, logarithmically growing, semiconfluent cells were dispersed with trypsin, and 1×10^6 cells were collected by centrifugation, resuspended in 700 μL of serum-free medium, and electroporated immediately after addition of the linearized plasmid to limit DNA degradation. To this end, a Bio-Rad gene pulser was adjusted to the settings indicated in Table 1. Electroporated cells were kept for 10 min on ice and then seeded on four 100 mm dishes in serum-containing medium. Selection medium with 1 mg/mL G418 was added 24 h after electroporation.

Infection. Infectious retroviral particles were generated after transfecting pM5βgeo under stable expression conditions into ψ-2 packaging cells. Virus-containing supernatants of these cells were used to infect BHK-A cells by applying conditions that rendered one in 4500 cells resistant. Such a low titer reduces the risk of multiple infections in a given cell (*5*).

Reporter Assays. β-Galactosidase (MUG Assay). Cells to be assayed for β-Gal activity were washed with PBS and harvested with TEN [40 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 150 mM NaCl], centrifuged (5 min at 200*g*), and resuspended in 200 μL of 0.25 M Tris-HCl (pH 7.8). Cell lysates were generated by three successive cycles of freezing and thawing, followed by centrifugation at 13700*g* at 4 °C for 5 min and collection of the supernatant fraction. β-Gal enzyme activity in lysates was determined by hydrolysis of the β-Gal substrate 4-methylumbelliferyl-D-galactoside (MUG) using 30 μg of MUG per milliliter in 200 μL of substrate buffer [60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM 2-mercaptoethanol]; fluorescence was measured at 5 min intervals, using a Millipore Cytofluor microtiter plate reader with excitation at 360 nm and emission at 460 nm. Fluorescence of each sample was measured in triplicate, corrected for the cell number, and standardized by referring to the mean of each series of measurements.

β-Galactosidase (in Situ Staining). Cells were washed with PBS and covered with fixing solution (0.1% glutaraldehyde and 2% formaldehyde in PBS) for 2 min, washed twice with PBS, covered with staining solution [5 mM $K_3Fe(CN)$, 6.5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, and 100 μg/mL X-Gal], and incubated overnight at 37 °C. After 20 h, blue, β-galactosidase-expressing cells were counted and referenced to the total number of cells. A 10% share of white cells which is clearly recognized in a background of blue (expressing) cells was taken to indicate instability.

β-Galactosidase (FACS Analysis). Viable cells were analyzed as described previously (*14*). Nonconfluent cells from a 9.1 cm^2 dish were washed with PBS, trypsinized, and suspended in PBS/10% FCS. After centrifugation (5 min at 200*g*), cells were dispersed in PBS/10% FCS at a density of 10^7 cells/mL. One hundred microliters of this suspension was transferred to a FACS tube, kept at 37 °C, and supplied with 100 μL of a prewarmed (37 °C) aqueous solution of 2 mM fluoresceine di-β-galactoside (FDG; prepared from a 100× stock solution in 1:1 H_2O/DMSO). After 1 min, the reaction was stopped by the addition of 1 mL of ice-cold PBS/10% FCS. Cells were kept on ice until the test. FACS analyses were performed at an excitation wavelength of 488 nm.

We also tried to use FACS to circumvent G418 selection. With this approach, we hoped to overcome the limitations of the selection drug which abolishes clones with a subthreshold expression level. These efforts were not successful since only high expressers produced a signal which was sufficiently separated from the background of nontransfected cells. This background was ascribed in part to a low level of endogenous galactosidase activity but, more importantly, to the fact that the detection procedure leads to some leakage of the protein and/or fluorochrome (*14*). We verified this explanation in a model experiment by mixing expressers and authentic (i.e., nontransfected) nonexpressers at various

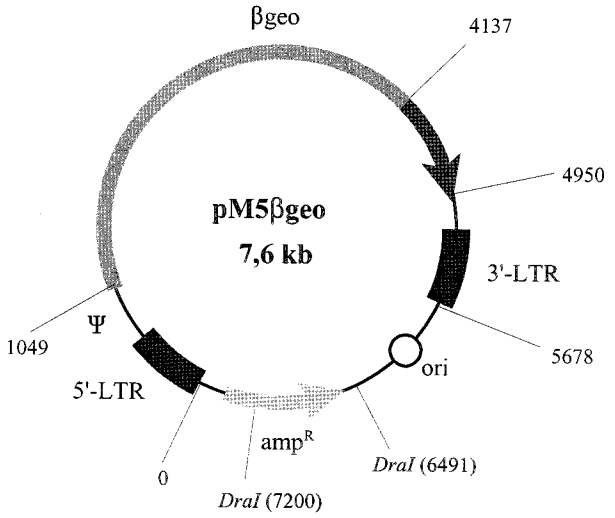

FIGURE 1: pM5β-geo, an expression plasmid for transfer by electroporation and retroviral infection. β-geo is a fusion gene comprising the sequences for β-galactosidase (gray) and neomycin resistance (black), driven by the 5′-LTR. For electroporation, the plasmid was linearized and depleted of plasmid sequences by *Dra*I digestion.

proportions. Sorting after the addition of FDG under hypotonic conditions generated a signal for the nonexpressing population which was shifted according to the proportion of expressing cells. These properties explain the relative position of the signal for shutoff clones (cf. clones I-18, E-2h, and E-3h in parts B and C of Figure 5).

Copy Number Determination. The presence of single copies was established by Southern blotting. Criteria that were used included the presence of a unique bordering fragment (starting in the integrate and terminating in the genomic DNA next to the integration site) and the absence of a transition fragment (starting in the integrate and ending in its next neighbor) which would be characteristic for tandem integration events (Figure 2).

Genomic DNA. Genomic DNA was prepared according to the method of Ramirez-Solis (*15*) with minor modifications. Southern blot analysis followed standard procedures as described in ref *16*.

Reactivation of Expression. To inhibit CpG methylation, clones were exposed for 72 h to medium containing 4−24 μM 5-azacytidine (5-azaC). Trichostatin A (TSA) was applied at a concentration of 165 nM in medium for 48 h to inhibit histone deacetylation. After this period, the percentage of expressing cells was controlled by β-galactosidase in situ staining. Expression levels were determined by MUG assays and referenced to the initial values prior to inactivation.

RESULTS

Experimental Strategy. For a strict comparison of transcription properties for proviruses which have been established by the two alternative gene transfer routes, we have developed an expression vector which is expected to have a comparable constitution after infection or electroporation. Our vector pM5βgeo (Figure 1) is a derivative of construct pM5SEPA which has been used extensively by Mielke et al. (*5*) for the molecular characterization of retroviral integration sites. Both vectors contain the long terminal repeats (LTRs) and the packaging signal of the murine myeloproliferative sarkoma virus (MPSV) but carry different combinations of reporter and selector genes. While pM5sepa is based on a secretory alkaline phosphatase and a puromycin resistance cistron, linked together by an internal ribosomal entry site (IRES), pM5β-geo carries a single transcription unit containing the sequences for the neomycin resistance gene and a β-galactosidase reporter (*17*). The advantage of the β-galactosidase reporter is its compatibility with a variety of sensitive enzyme assays and with single-cell analyses by fluorescence-activated cell sorting (FACS). Since FACS signals were not adequate for an efficient recovery of transfected cells (see Materials and Methods), the neomycin resistance function had to be used to this end. This marker mediates resistance at very low expression levels, and there are reported cases where the β-geo fusion protein rendered cells resistant at a transcriptional rate even below the detection limit of the associated β-galactosidase activity (*17*).

To closely match the states after electroporation and infection, electroporation experiments were performed with a template for which the two *Dra*I sites at the ampicillin resistance gene were used for linearization and the removal of major parts of plasmid sequences. For obvious reasons, the extent of excision has to represent a compromise between an optimum protection of the expression unit from nucleolytic processes and a comprehensive removal of prokaryotic sequences. Under the conditions specified above (short residence time of vector DNA in serum-free medium prior to electroporation), no alterations of the transcription cassette were observed.

Generation of Single-Copy Clones by Retroviral Infection and Electroporation. Present retrovirus-mediated gene transfer techniques involve a two-step procedure, i.e., (i) use of a packaging cell line (instead of a helper virus; see ref *7*) for the generation of infectious particles and (ii) use of cell supernatants for the infection of BHK-A cells with the recombinant virus particles. Analysis, by Southern blotting, showed that, out of 24 clones, 19 contained a unique bordering signal typical for a single-copy integration event. This signal is generated by *Sph*I cuts at a defined site within the neo^r part of the vector and at the nearest genomic *Sph*I site (see Figure 2A). Some clones (marked by stars in Figure 2B) contained a weak additional signal, and this was also true if a corresponding left bordering fragment was visualized with a β-galactosidase probe (not shown). Since the strength of the two signals varied widely, they indicated the presence of clonal mixtures rather than the independent integration of a second copy. This assumption was verified by subcloning (not shown).

In a number of pilot experiments, the electroporation conditions were varied with regard to DNA input concentration, voltage, and capacity to optimize the conditions for single-copy integration events (see Materials and Methods). Table 1 indicates that as much as 37% of the single-copy clones can be obtained at a low DNA concentration under conditions where copy numbers were found to be rather independent of the electrical settings. Figure 2C shows analyses on 27 clones in which case 6 harbored a single copy of the transgene.

Expression Levels. Seventeen infectants were used, and 21 transfectants with a single-copy integration event were collected from gene transfer experiments 1−4 (Table 1). For these clones, expression levels were determined using the

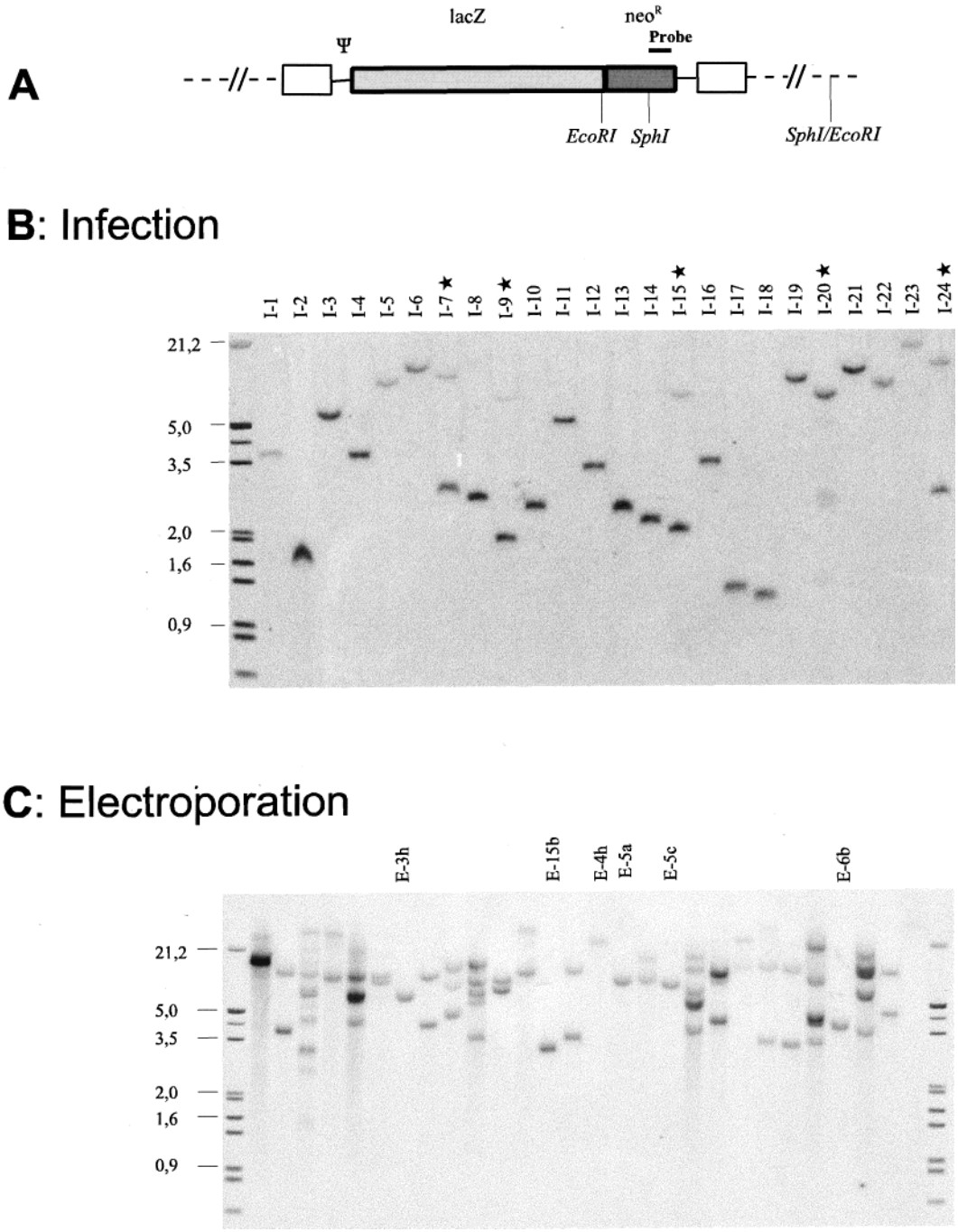

FIGURE 2: Status of the transgenes after transfer by infection (B) and electroporation (C). (A) State after integration of linear pM5β-geo after infection or electroporation. (B) Southern blots on genomic DNA from infected cells cut by *Sph*I and probed by the indicated neo^r probe. Unique bordering fragments indicate single-copy integration events. Two bands in five lanes (stars) are typical for mixtures of two clones rather than the simultaneous integration of two copies (see the text). (C) Southern blots on genomic DNA from electroporated cells cut by *Eco*RI and probed by the indicated neo^r probe. The six marked clones generate a unique bordering fragment, and four of these are among the 21 single-copy clones used for expression analyses.

conversion of the fluorigenic substrate 4-methylumbelliferyl-β-D-galactoside (MUG) by cellular extracts as a sensitive enzymatic test. Figure 3 shows the transcriptional properties, arrayed according to expression levels, for the infectants (black bars) and the transfectants (white bars). Although the three highest expressing clones originate from infection and the two lowest expressers are transfectants, average levels of expression differ by a factor of only 1.3. This corresponds to our preliminary observations with low-copy number transfectants (*5*) but is clearly different from the dramatic (10−50-fold) differences reported previously (*7*).

Long-Term Stability. The single-copy infectants and transfectants were cultivated for several months in the presence or absence of the selection drug G418 to examine the persistence of expression levels. At certain intervals, the clones were stained with X-Gal and the proportion of blue (expressing) and white (shutoff) clones was determined. While in the presence of G418 all cells remained active as expected for a fusion protein with linked reporter and selector functions, clones kept in the absence of selection pressure exhibited marked differences depending on the gene transfer method. Figure 4A shows that in the absence of selection

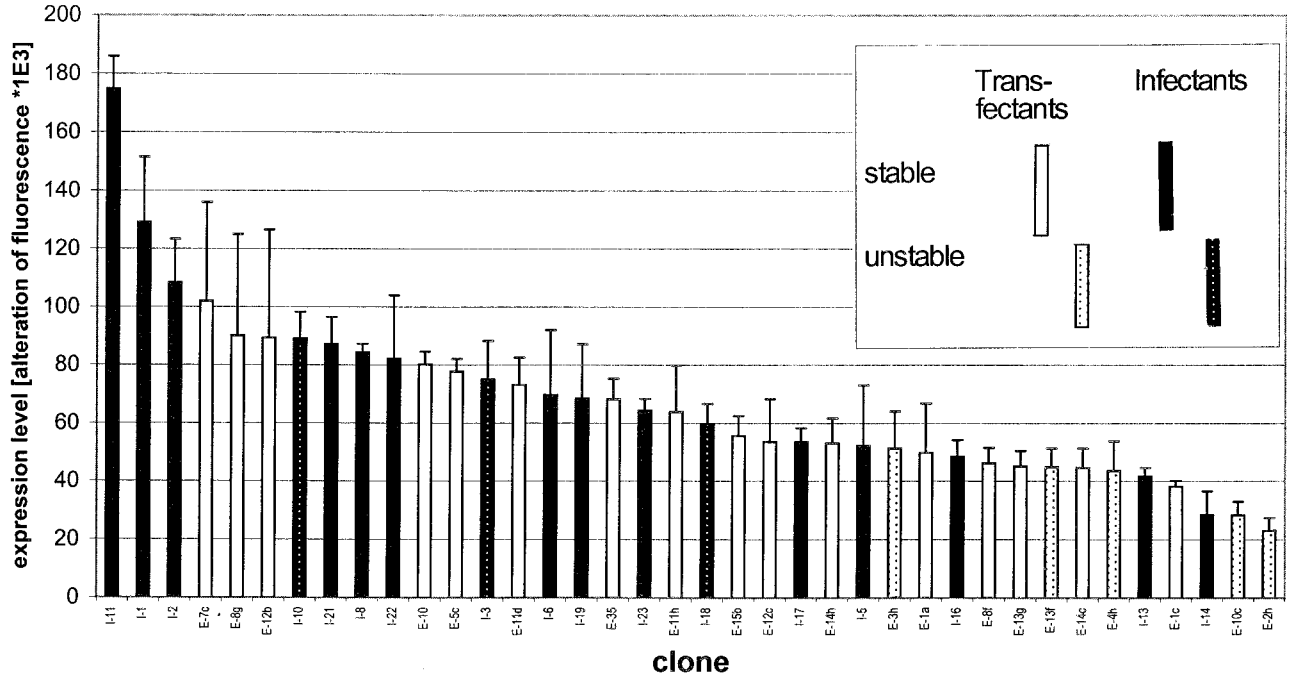

FIGURE 3: Expression levels for single-copy infectants (black bars; prefix I) and transfectants (white bars, prefix E). Activities of the β-galactosidase reporter were quantified on cell extracts using the MUG assays and given as fluorescence change per 10^6 cells in 5 min. Clones which proved to be unstable upon long-term passage have been denoted with dots (see also Figure 4). Note that all unstable transfectants belong to the low expresser class.

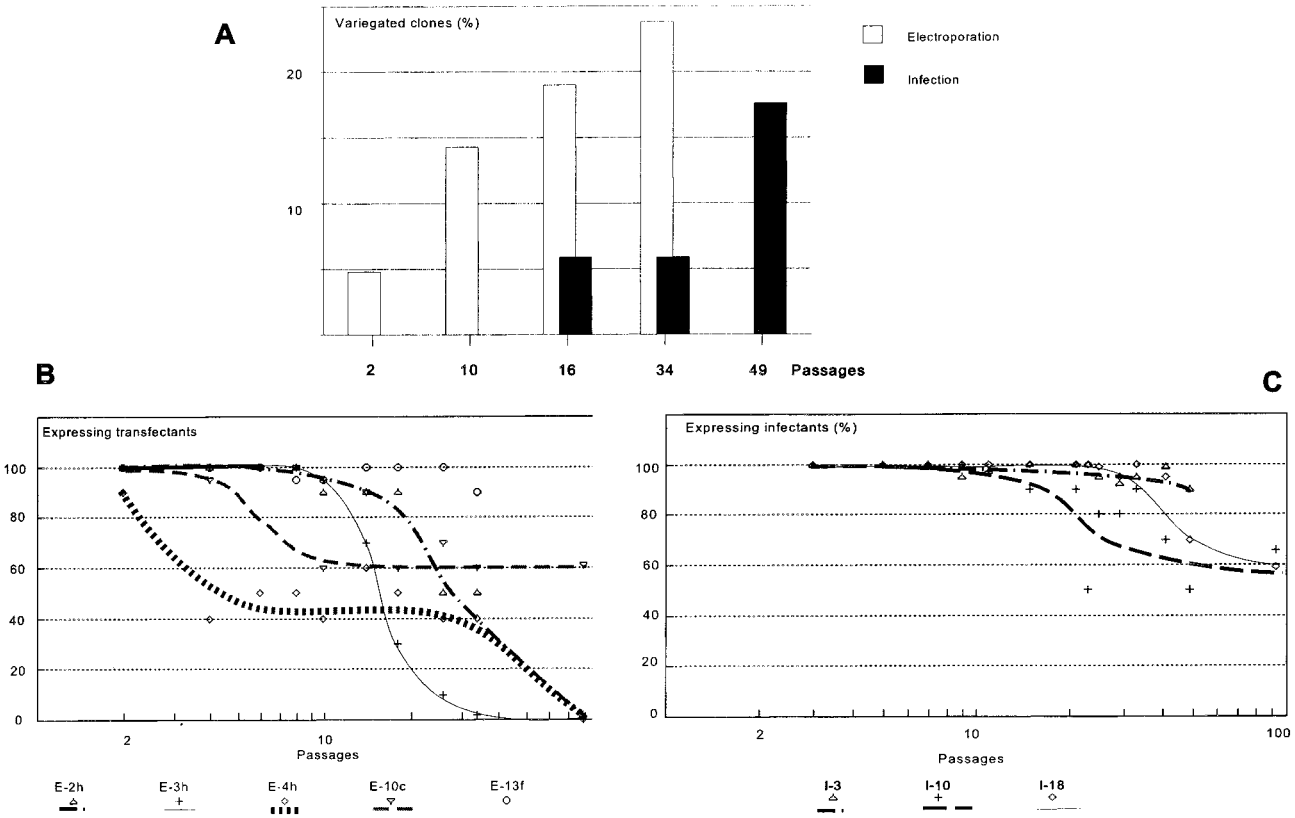

FIGURE 4: Decay of expression for single-copy infectants (black bars) and transfectants (white bars) with time. (A) After release of the selective pressure, clones were cultivated for many passages. At certain intervals, unstable clones were identified by a blue-white screening whereby clones containing >10% of cells not staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) were classified unstable. (B) Expression decay curves for the transfectants from panel A, plotted on a logarithmic time scale (passage number). (C) Expression decay curves for the infectants from panel A (plotted as described for panel B).

pressure (G418) the first transfectant loses expression at passage 2. At passage 34, where there is no more than one unstable infectant, there are already five (24%) unstable transfectants. At passage 49 (about 250 population doublings), variegation is observed for only three out of 17 infectants, meaning that this group of clones remains essentially stable for the purpose of almost any application. Among the transfectants there exists a clear-cut correlation

between their initial expression level and the probability of losing expression. We have marked the unstable clones in Figure 3, and it is clearly seen that all unstable *transfectants* are clones with a low initial level of expression.

For a study of the inactivation process, the unstable transfectants were cultivated up to passage 79 (Figure 4B) and the variegating infectants up to passage 94 (Figure 4C). At this point, another difference between the two groups emerged with regard to the proportion of nonexpressing cells within an unstable clone. Figure 4C indicates that all three infectants still contain more than 50% of expressing cells at passage 94 whereas three out of five unstable transfectants were totally white (E-2h, E-3h, and E-4h). These data indicate that, for BHK-A cells, the process of retroviral infection places the provirus into a genomic environment which is permissive for stable expression whereas electroporation also populates sites that are less intrinsically stable. Alternatively, the enzyme (integrase)-catalyzed insertion process, which introduces a staggered cut and is accompanied by only minor changes (duplications) of flanking genomic sequences, preserves the features of the integration site to a greater extent than the illegitimate recombination event which integrates the electroporated DNA, probably at pre-existing single-stranded breaks in the genome (*18*). In some cases, subsequent genomic instability could be ascribed to the structural features of these sites (*19* and references therein).

Inactivation Patterns. The loss of expression among the transfectants is more frequent and occurs earlier than for the infectants. In principle, two modes of inactivation have to be distinguished: (i) a decreased level of expression in all cells leading to a single band with an intermediate position and (ii) a variegated expression in which a gene is completely silenced, but only in a proportion of cells in the derivatives of a clone. Our observation that white cells coexist with blue ones clearly favors the second possibility. This could be quantitatively established by a FACS analysis using fluoresceine di-β-galactoside. FDG is another fluorigenic substrate which can be transferred into living cells via hypotonic shock. Figure 5 demonstrates the distribution of cells which have been kept for 35 or 51 passages with or without selection pressure. In both cases, a clear bimodal distribution of cells is caused by populations which are either positive or negative for β-galactosidase.

Parts B and C of Figure 5 seem to indicate some remaining activity after shutoff since the corresponding signal is not coincident with the control (Figure 5A), but this is clearly not the case; we have shown that this phenomenon is a consequence of the required hypotonic shock which not only mediates cellular uptake of FDG but also causes some leakage of the fluorescent product and possibly β-galactosidase which in turn adhere to the shutoff clones (see Materials and Methods).

DISCUSSION

In this study, we have compared the level as well as the long-term stability of expression for single-copy proviral templates which have been transferred either by the specific process of retroviral infection or by electroporation. We have performed these experiments with linearized DNA largely devoid of vector sequences and in the absence of carrier DNA, i.e., under conditions which guarantee that expression characteristics reflect the transcriptional properties of the genomic integration site. Thereby, we have minimized the possibility that transcriptional properties are affected by the presence of major parts of foreign DNA or by the random opening of a circular construct. We have given preference to gene transfer by electroporation rather than Ca phosphate-mediated transfection. Electroporation generates a major proportion (up to 37%; Table 1) of cell clones with a single copy of the transgene, whereas Ca phosphate typically yields between five and several hundred copies, mostly in the form of head-to-tail concatenates (*18*). Under these conditions, we could not confirm the conclusion by Hwang and Gilboa (*7*), who found that the expression of single-copy genes introduced by retroviral infection is significantly more efficient than that of transfected genes. Our experiments yield only a slightly (1.3-fold) better average transcription rate for infected genes (Figure 3), and this is in close agreement with an earlier study which had compared infectants with low-copy clones, established by a standard electroporation protocol (protocol 1 in Table 1; see ref *5*).

The extreme difference in transcriptional activity of $CaPO_4$-transfected and infected constructs described previously (*7*) can be explained with at least two different ways of reasoning.

(i) The first is the fact that for previous work circular vectors were transfected and therefore contained an unspecified kind and length of prokaryotic vector DNA on both sides due to random opening. Vector DNA might impair transcription (*7*, *20*) since it is generally GC rich and thereby a major target for de novo methylation.

(ii) The second is by principal differences between the Ca phosphate and electroporation pathways of transgene integration. A prominent characteristic of the Ca phosphate method (but not of electroporation; see Table 1) is the occurrence of tandemly arrayed multicopy integrates (concatemers). Although the routes of DNA transfer differ fundamentally between these transfection techniques (*21*, *22*), these differences are probably less important than the local concentration of DNA which is established prior to integration. Folger et al. (*23*, *24*) have demonstrated, by microinjection, that single linear molecules become inserted through their ends. When more than five molecules were injected, they formed the typical head-to-tail array by recombination (rather than replication). If it is assumed that the few single-copy clones obtained from Ca phosphate-mediated gene transfer arise as a consequence of a rather occluded genomic site which does not invite the entry of multiple copies by recombination, then the selection for these rare events would mean enriching mediocre (occluded) loci. Therefore, only electroporation would lead to a proportion of single-copy clones that can be considered representative for the entire spectrum of available sites.

For the present series of infectants, there was a factor 6 difference between the highest- and lowest-expressing clones and a factor 4 difference for the transfectants using the highly sensitive neo^r selection marker. This rather narrow range resembles previous reports derived for NIH 3T3 murine fibroblasts after puromycin (*5*) or hygromycin selection (*25*), and for BHK-A cells after G418 selection (*26*). The data summarized in Figure 3 prove that the range of high producers is only slightly more populated by the infectants. Similar levels of high expressers might simply arise by

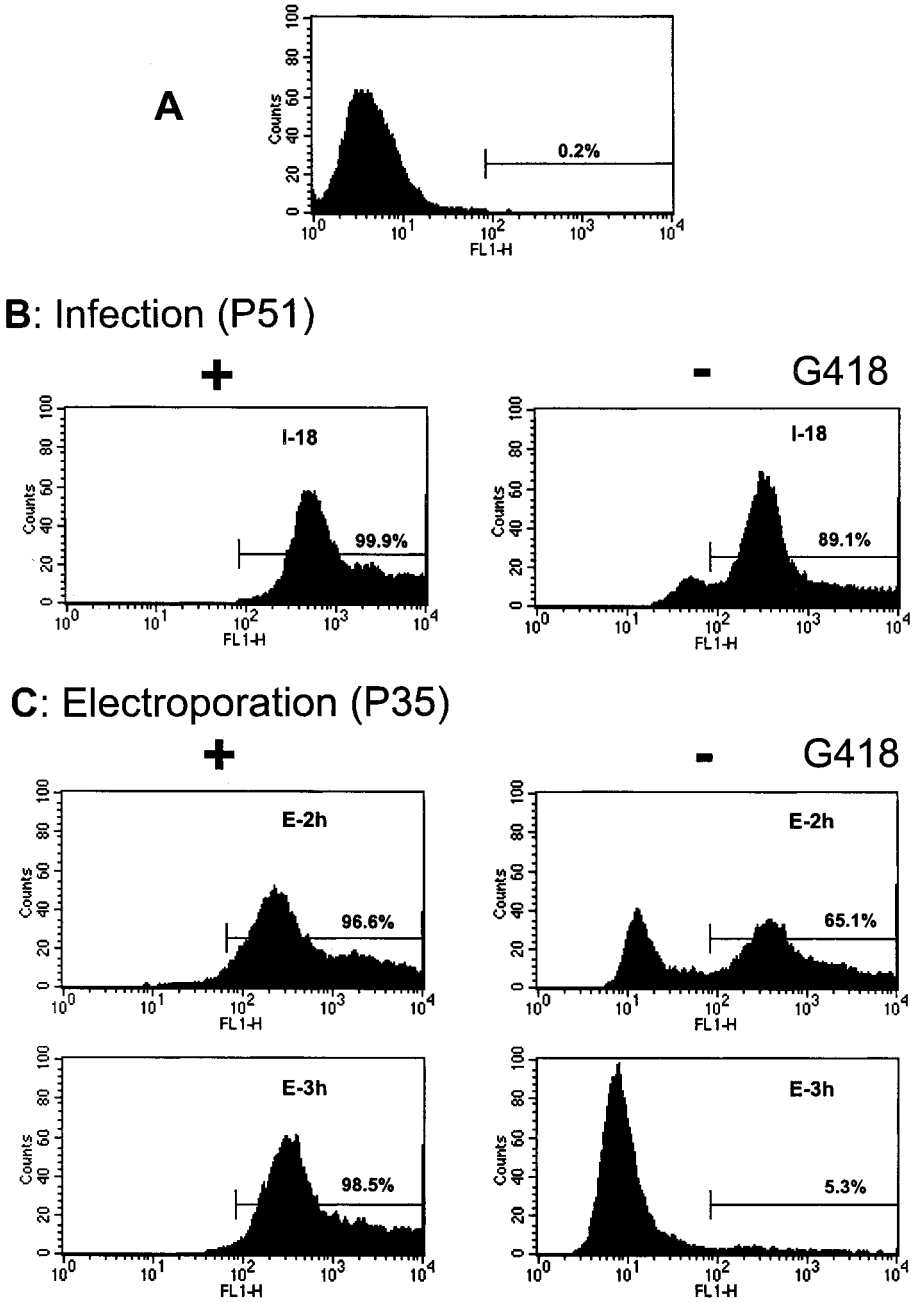

FIGURE 5: Shutoff patterns analyzed by FACS-scan analysis. (A) Nontransfected control in the presence of the fluorigenic dye (FDG). (B and C) Behavior of unstable clones, exemplified by clone I-18 (infection) and clones E-2h and E-3h (electroporation). The infectant was cultivated for 51 passages, and the transfectants were cultivated for 35 passages in the presence or absence of the selective drug G418.

chance or because of distinct features which are common to a certain class of sites which are targeted by illegitimate recombination, be it retroviral infection or transfection (*1*, *27*). De Ambrosis et al. (*28*) have characterized these targets as "sites of chromosomal fragility" or "recombination hotspots" with attributes such as hypersensitivity to nuclease attack, the phenomenon of bending, and the propensity to form DNA superstructures (*1*, *5*, *27*, *29*). All these features are also common to scaffold/matrix-attached regions which have been implicated in genomic deletions and which we have found to invite retroviral integration events (*5*).

A marked difference between the two gene transfer routes becomes evident only during a long-term culture (Figure 4A). While the first transfectants exhibit reduced levels of expression, only a few passages after removing the selective pressure, all infectants remain stable up to passage 16, and thereafter, they develop instability at a slower rate. After cultivation of these unstable infectants and transfectants for an additional 45 passages (about 230 population doublings), most cells within the infected clones still expressed the reporter (Figure 4C) while three out of five transfectants had completely lost their expression ability (Figure 4B). Consequently, not only the probability to lose expression but also the extent of silencing in the unstable clones is dependent on the gene transfer method. It is notable that, without exception, all unstable transfectants represent clones that were low producers initially. Instability may therefore be the property of a class of target sites either which is surrounded by repressive chromatin or which has undergone extensive rearrangements during the integration step.

What is the mechanism underlying transcriptional inactivation? A Southern blot analysis revealed that the clones in

Figure 4 contained the intact gene at passage 34 or 49, whereas one clone (E-4h) lost the transgene later (not shown). For infectants, the loss of expression has frequently been ascribed to epigenetic inactivation of the promoter. Previous studies have demonstrated that retroviral sequences cause the silencing of transgene expression in mice (*30*), primary fibroblasts (*31*), and certain cultured cell lines (*32*, *33*). Sequences within the retroviral LTR seem to be one target for methylation (quoted in ref *34*) which may reflect part of a eukaryotic defense mechanism against retroviral infection. It should be noted, however, that silencing of transgenes has also been observed for constructs lacking any viral sequences. Therefore, it is yet unclear if these epigenetic inactivation events are triggered by particular sequences or if certain enhancer−promoter combinations have different abilities to overcome the negative effects of an integration site. Since for the present study electroporated copies mimic the structure of the provirus, differential silencing cannot be caused by a selective recognition and inactivation of particular sequences but rather by the structure and/or location of a given integration site. Integration site-dependent silencing of gene expression has been detected in proviruses as well as in transfected genes in mammalian cell culture (*35*, *36*). This phenomenon resembles "position-effect variegation" in *Drosophila* and transgenic mice (*37*, *38*).

Besides CpG methylation, another prominent mechanism of epigenetic inactivation is histone deacetylation. Recently, these two processes have been dynamically linked; the methyl-CpG-binding protein MeCP2 appears to reside in a complex with histone deacetylase and can mediate formation of transcriptionally repressive chromatin on methylated promoter templates in vitro (*39*, *40*). Once a given site becomes fully occluded and has reached a certain density of methylation events, these steps cannot be reversed by inhibitors of DNA methylation and histone deacetylation (*41*). In accord with these findings by Lorincz and colleagues, we found a reactivation of partially but never of fully inactivated single-copy clones by 5-AzaC, TSA, or both. In striking contrast, a completely silenced multicopy clone could be fully reactivated by the action of TSA (unpublished data). While these observations suggest mechanistic differences for repeat-induced (*8*, *42*) and spontaneous shutoff events, more work will be required to establish the relative role of methylation and deacetylation events for various inactivation pathways.

From a practical viewpoint, the most important conclusion from our study is the fact that highly producing cells can be derived both by retroviral infection and by electroporation protocols favoring single-copy integration. While clones generated by retroviral infection are largely stable under the conditions described here, the most highly expressing 70% of single-copy transfectants have similar properties. Electroporation therefore emerges as a practicable alternative if the infection pathway is to be avoided for reasons of biological safety. The recent demonstration that skeletal muscles of small animals can be genetically modified by the electrotransfer of plasmids which are subsequently expressed for more than a year (*43*) shows that this method is not restricted to cultivated cells.

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