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Lack of Correlation between Basal Expression Levels and Susceptibility to Transcriptional Shutdown among Single-Gene Murine Leukemia Virus Vector Proviruses

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Integrated retroviruses or retroviral vectors may be transcriptionally inactive although their promoter-enhancer regions are able to direct transcription in the host cell. We have used single-gene retroviral vectors with a long terminal repeat-directed *neo* marker gene to determine if the level of transcription relates to the susceptibility of a provirus to inactivation. We used two isogenic vectors, carrying long terminal repeats with a strong and a weak transcriptional enhancer derived from SL3-3 and Akv murine leukemia viruses, respectively. Nonselected cell clones of the murine lymphoid cell line L691 with single integrated vector proviruses exhibiting a 20-fold range of initial expression levels were studied. The basal expression level of a given cell clone with a single provirus did not show any pattern of correlation with the long-term stability of expression, as monitored for periods up to 150 days. Our results thus indicate that the inactivation mechanism operates independently of the initial transcriptional activity of the provirus.

Proviruses of noncomplex retroviruses are transcriptional units whose promoter-enhancer strength is determined by the interaction of *trans*-acting cellular factors with the *cis*-acting viral promoter-enhancer sequences. An integrated provirus with an intact promoter-enhancer region is, however, not necessarily transcriptionally active. The overriding negative effect that may lead to complete shutoff of transcription of an integrated provirus has been observed in a variety of systems. For murine leukemia virus-derived vectors, the negative effect has been detected either in cultured cells with variation among transduced cell clones (16, 32) or after transplantation of cultured cells into animals (24). Other examples include endogenous proviruses of mice (2, 17, 29) and chicks (6) that are transcriptionally silent but, once activated, are fully replication competent. Another model for provirus inactivation is based on Rous sarcoma virus in rat fibroblasts, in which the vast majority of integrated proviruses are silent (12, 13), although the Rous sarcoma virus promoter-enhancer supports transient expression in rat fibroblasts.

In some cases, the site of integration may play a role in transcriptional inactivation of a provirus (12, 14, 16, 30). Not only the stability of expression but also the level of provirus transcription is believed to be influenced by the site of integration. The effect on the expression level is most likely due to enhancer-like activities that may happen to be located round the provirus integration site (15). We have found stimulatory effects of this type to be particularly pronounced for proviruses carrying a weak transcriptional enhancer (8, 26).

With the two types of proposed effect, on the level and stability of expression, the question arises as to whether one is influenced by the other. Previous studies of stability have mostly employed cell clones derived after selection for vector expression, using a selectable marker driven either by the long terminal repeat (LTR) or by a second promoter in the vector (16, 32). While the use of cell clones selected for LTR-driven expression may preclude analysis of the full range of vector expression levels (13, 27), two-promoter vectors may introduce problems associated with promoter interference (10, 11).

From a series of studies of the expression of nonselected Rous sarcoma proviruses in Rat-1 cells, Lang et al. have proposed that a diminution of proviral expression may represent a first step toward transcriptional inactivation (19). By analogy, one possibility is that also among identical proviruses integrated at different sites, those with the lowest expression levels are most susceptible to inactivation.

In this study, we used a set of cell clones, each carrying a single integrated retroviral vector provirus (26), to look for a possible correlation between initial expression level and susceptibility to shutoff of expression.

To preserve the full variation among the proviruses in terms of expression level and stability, the cell clones used were obtained without any selection for vector expression. In addition, two isogenic vectors differing only in the enhancer region were used (26). We have analyzed the long-term stability of expression of 21 proviruses in individual cell clones and found that some of the proviruses gradually lose expression, albeit with a rate that varies among clones. The results presented here do not point to any simple correlation between the susceptibility of a provirus to transcriptional shutdown and its initial expression level as determined either by clonal variation among identical proviruses or by differences in provirus enhancer strength.

MATERIALS AND METHODS

Vectors. The retroviral vectors Akv-neo and SL3-3-neo (Fig. 1) have been described previously (26).

Cell lines. L691 is a non-retrovirus-producing cell line derived from a radiation-induced thymoma of a C57 mouse (22). Cell clones derived from a population of either Akv-neo- or SL3-3-neo-transduced L691 cells and isolated without selection have previously been described (8, 26). Single-vector-copy cell clones from these populations are called A*n* and S*n* for clones with Akv-neo and SL3-3-neo, respectively. By use of

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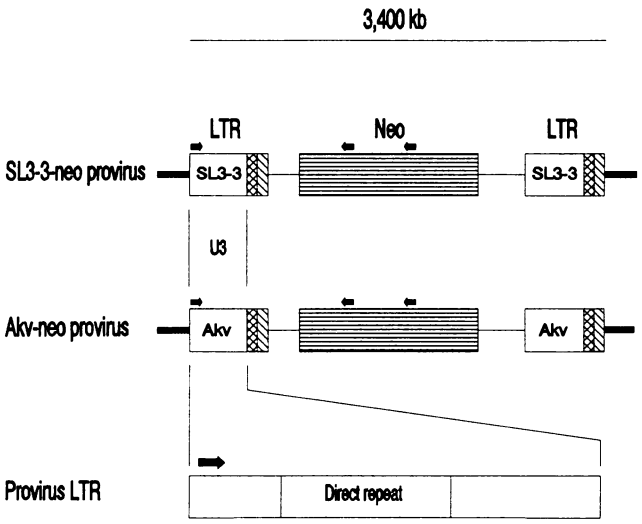


FIG. 1. Schematic representation of the vector proviruses Akv-neo and SL3-3-neo. Box with horizontal lines, Tn5 sequences including the *neo* gene; cross-hatched boxes, R region of the LTR; hatched boxes, U5 region of the LTR; open boxes, U3 region of the LTR; thin line, other virus sequences; thick line, host DNA flanking the integrated vector provirus; horizontal arrows, oligonucleotides used for PCR amplification of the integrated vector provirus. At the bottom is a magnified representation of the U3 region for both vectors. "Direct repeat" represents the enhancer region of the two viruses, which, except for one base pair position upstream in U3, is the only part of the two vectors that is not identical (26).

a vector rescue assay, it was confirmed that the population used for isolation of cell clones was free of helper viruses.

To ensure that no mutations that might influence the level and stability of expression had been introduced, the enhancer region, the direct repeat in the U3 region, of all of the proviruses was sequenced directly by PCR techniques (28), using the primers depicted in Fig. 1. No major rearrangements or point mutations were found (8). Furthermore, RNase protection analysis of the vector transcripts in some of the cell clones confirmed the use of proper initiation and polyadenylation sites (26).

Cell culture. Cells were grown in suspension in RPMI 1640 medium–10% newborn calf serum. G418 was from Sigma Chemical Co., St. Louis, Mo.

Determination of vector expression levels. Vector expression levels were determined at the RNA or protein level as *neo*-specific RNA or neomycin phosphotransferase II (NPT II) activity, respectively. The two assays show full proportionality, but the RNA measurements are less sensitive than the enzyme measurements.

Vector-specific RNA levels were determined by dot blot hybridizations as previously described (8), using ^{32}P-labeled DNA hybridization probes for *neo* (3) and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The hybridization signals were quantitated densitometrically on an enzyme-linked immunosorbent assay reader (405 nm), using the absorption of the film outside the dots as the blank. This allows a more accurate quantification than scintillation counting of the hybridized probe (9). Only exposures in the range of proportionality between radioactivity and film absorption were used.

Specific NPT II activity was determined as described previously (9, 26).

DNA extraction and Southern blot analysis. High-molecular-weight DNA was extracted, and electrophoresis, blotting, hybridization, stripping, and washing were done as described in the Zeta-probe manual. The *neo* probe and labeling procedures were as described previously (8).

RESULTS AND DISCUSSION

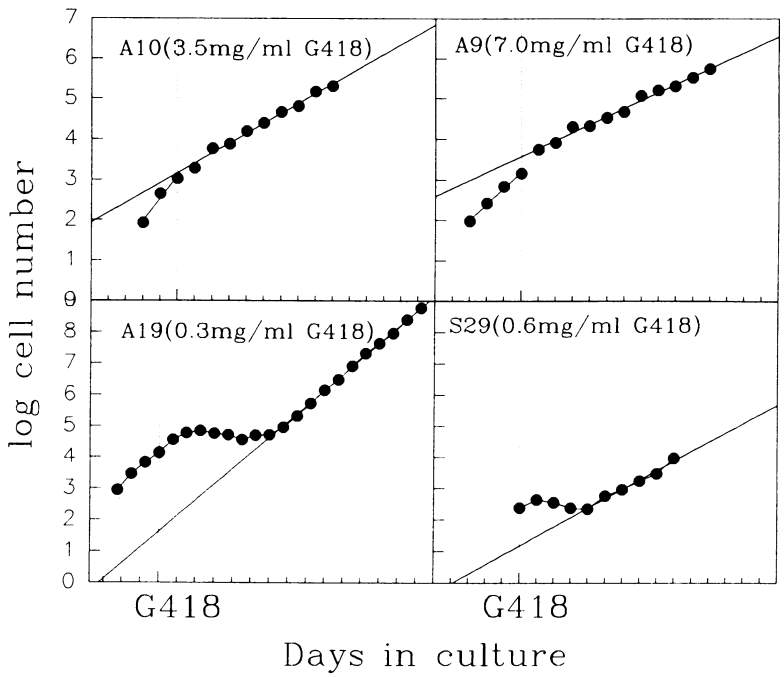
Choice of model system. We wanted to compare the susceptibility to transcriptional inactivation of closely related or identical proviruses with various initial levels of transcription. For this purpose, we chose material based upon single-gene retroviral vectors with an LTR-driven reporter, using two variant LTRs of different enhancer strength. We used two sets of unselected cell clones of the murine lymphoid cell line L691 transduced with a single copy of either Akv-neo or SL3-3-neo provirus (Fig. 1), harboring a weak or a strong enhancer, respectively (26). The collection contains proviruses covering about a 20-fold span of expression levels as well as proviruses devoid of expression.

For analysis of the stability of expression of the individual proviruses, the starting point must be a clonal culture in which all cells express the provirus (16). In initial experiments, we therefore tested the effect of short treatments at various concentrations of G418 on cell survival after cultivation of the cell clones. Two types of pattern were observed (Fig. 2). One type is illustrated by cell clones A10 and A9, which showed no signs of cessation of cell growth, but at the very high G418 concentrations used, a slight reduction in growth rate was observed from day 0, in agreement with our previous description of the effect of G418 selection on homogeneous cell populations expressing NPT II (27). For this type of cell clones, the specific NPT II activity is unaffected by growth in G418-containing medium, even for periods of up to 2 months in culture (7). Another type of pattern is illustrated by cell clones A19 and S29, which showed an initial break in growth upon G418 addition (Fig. 2). After some days, the cells returned to a growth rate similar to that found before selection. An increase in specific activity of NPT II after G418 selection of cell clones A19 and S29 (Fig. 2B) suggested that only a fraction of the cells at time zero produced sufficient NPT II to allow growth in G418-containing medium. For cell clone S29, the fraction of G418-resistant cells corresponds closely to the change in specific NPT II activity. For A19, a low survival may be caused by a toxic effect of heavy cell death in the culture, postponing reestablishment of exponential growth (1). These results indicate that cultures of the cell clones may contain a large fraction of cells that show no or only low levels of provirus expression.

To eliminate any non-NPT II-producing cells that had accumulated during cultivation, all cell clones were subjected to 1 week of mild selection with G418 at 0.4 mg/ml (the lowest concentration that kills all nontransduced L691 cells) immediately before monitoring of the stability of vector expression.

Vector expression during prolonged passage in cell culture. To determine the stability of expression from the individual vector proviruses in the two clone sets, 7 SL3-3-neo and 11 Akv-neo clones were kept for 144 days in cell culture in G418-free medium. Specific *neo* RNA levels were determined by dot blot hybridization analysis using a *neo* probe and a GAPDH reference probe. In Fig. 3, relative *neo* mRNA levels for the SL3-3-neo cell clones are shown as a function of days in culture. The same experiment and calculations were done for the Akv-neo clone set (data summarized in Fig. 6; see below). While the measurements are compatible with a completely

A



B

	Specific NPT II activity before selection	Specific NPT II activity after selection	Specific NPT II activity before/after selection	Fraction of G418 resistant cells	Regression coefficient Fig. 3.
A19	0.025	0.50	0.05	0.0032	-0.28
S29	0.26	3.0	0.08	0.0625	-0.30

FIG. 2. Effect of a short G418 selection step on cell clones harboring an integrated vector provirus expressing the neomycin resistance gene. (A) Effect of G418 addition on cell growth; (B) effect of G418 selection on specific NPT II activity and on cell survival. At day 0 (thin vertical line; vertical marks on the *x* axis represent 1-day intervals), selection was imposed upon exponentially growing cultures of four clones (A10, A9, A19, and S29), using the indicated concentrations of G418 in the medium. For clones A19 and S29, the fraction of cells surviving G418 selection has been estimated by extrapolating the exponential growth curve reappearing after selection back to time zero. The straight line is drawn on the basis of a linear regression analysis, and its interception with the *y* axis at day 0 was used to estimate the fraction of G418-resistant cells in the two cultures at the day of application of selection (interception of regression line with *y* axis/total number of cells at day 0) as given in panel B. The specific NPT II activity of cultures (counts per minute per microgram of protein relative to the amount in a standard cell extract included in all sets of experiments) before and after selection was determined as described previously (9).

stable expression pattern for some of the clones, others lost expression with time, albeit at different rates.

For all cell clones showing decreasing vector transcriptional activity as a function of time in cell culture, Southern blot analyses were performed with samples taken at time zero and after 144 days in culture, using a *neo* probe to detect vector sequences and a mouse genomic probe for standardization. No significant loss of vector sequences could be detected in any of the cell clones by Southern blotting analysis (data not shown).

Stability of three inactive SL3-3-neo cell clones. From the population of unselected L691 cells transduced with SL3-3-neo vector provirus, three cell clones (S5, S20, and S55) lacked vector expression as analyzed by RNA dot blot or NPT II enzymatic activity (26), although their proviruses appeared physically intact by PCR and sequence analyses (data not shown). It was therefore of interest to examine whether these three proviruses could be transcriptionally activated and, if so, to examine their stability of expression.

Cultures of the cell clones S5, S20, and S55 were treated with 5-azacytidine, a demethylating drug that in several cases has been reported to activate silent proviruses (16, 31). After 3 days of cultivation in media with 5-azacytidine (5 μg/ml), the cells were subjected to G418 selection in the absence of 5-azacytidine. After 14 days of selection, one S5-derived, three independent S20-derived, and four independent S55-derived subpopulations were obtained.

To study the stability of expression of the proviruses, the subpopulations were changed to nonselective conditions and monitored for *neo* expression (Fig. 4). The independent S20 and S55 subpopulations all showed rapidly decreasing vector transcriptional activity. The single S5 subpopulation also showed decreasing vector expression but to a lesser extent.

To ensure that the decreasing transcriptional activity of these vectors was not caused by loss of vector sequences, Southern blot analysis was performed. Figure 5 shows an autoradiogram of a Southern blot with the initial S5, S20, and

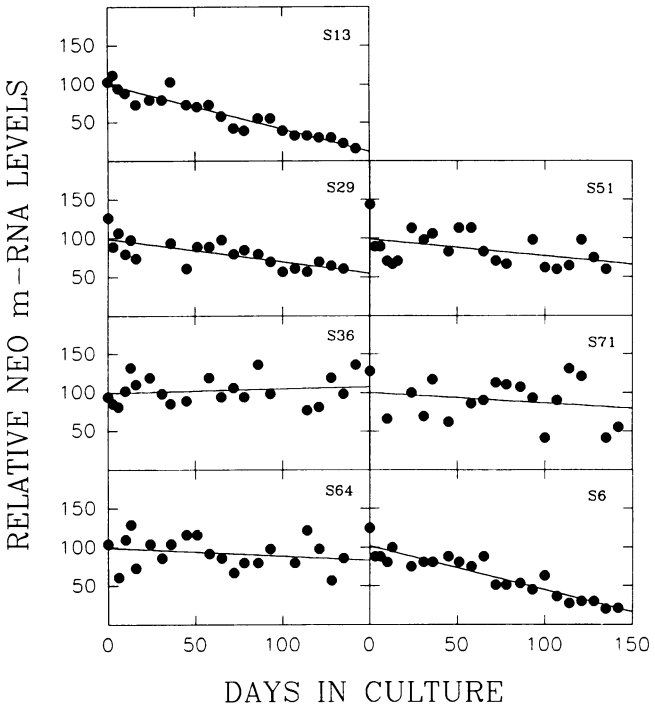


FIG. 3. Long-term vector expression in cell clones. Seven cell clones harboring a single integrated SL3-3-neo vector provirus were monitored for 144 days in cell culture in the absence of G418. Stability of vector expression for each cell clone was monitored by determination of the *neo* mRNA content in crude RNA samples taken at the indicated intervals. Quantification was performed by RNA dot blot analysis (not shown) whereby a probe for the *neo* gene was used to estimate vector RNA level and a probe for the GAPDH gene was used to estimate total mRNA content in the sample. By scanning the autoradiograms, specific *neo* mRNA levels were determined as *neo* optical density at 405 nm/GAPDH optical density at 405 nm. After regression analysis of the curves, relative *neo* mRNA levels for all cell clones were set at 100 at the beginning of the experiment. Each value represents the average of two independent RNA sampling and hybridization experiments.

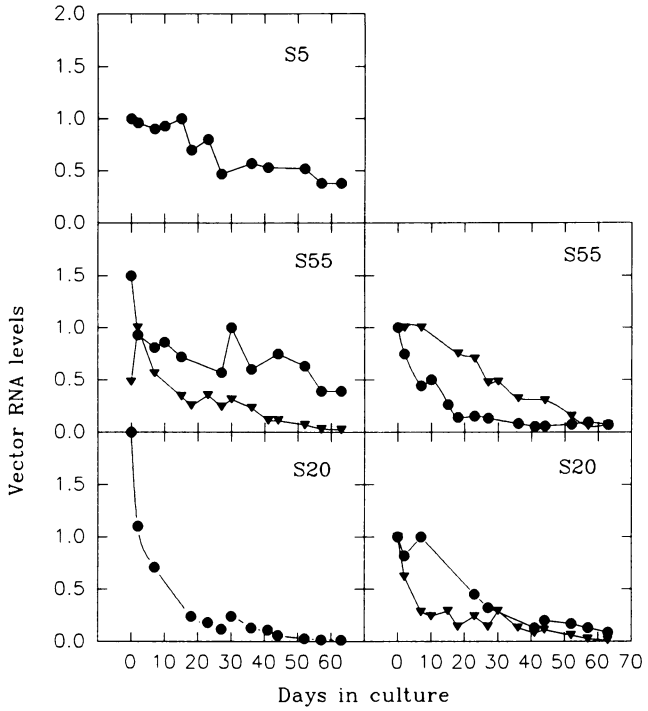


FIG. 4. Stability of vector expression in cultures derived from nonexpressors. After treatment with 5-azacytidine and selection for vector expression with G418 (see text), the resulting subpopulations were monitored for stability of vector expression as described in the legend to Fig. 3. The subpopulations were monitored for 64 days in cell culture, and specific *neo* mRNA levels are plotted by using the same relative scale for all subcultures.

S55 subclones and the subpopulations, after selection and after the 64 days in cell culture. No major gain or loss of vector sequences in any of the subpopulations had occurred, judging from the intensities of the bands of the autoradiogram (Fig. 5). We infer that the proviruses in these three cell clones are subject to rapid shutdown of expression.

No simple relationship between the level and stability of vector expression. The observed variation in susceptibility to transcriptional inactivation among a set of proviruses representing a range of expression levels made it of interest to determine if any relationship between the level and stability of expression existed. In Fig. 6, the regression coefficients calculated from Fig. 3 and from the similar analysis of the Akv-neo clone set are shown as a function of basal transcriptional activity (26) of the cell clones. The regression coefficient of a clone can be considered a measure of the stability of expression of the integrated vector under the given culture conditions. A high negative value indicates unstable expression, and a regression coefficient around 0 is compatible with a stable expression pattern. As can be seen in Fig. 6, both sets of cell clones exhibit long-term vector expression levels in the range from full stability to only a modest reduction in vector expression levels. Furthermore, there is no correlation between stability and basal transcriptional activity for any of the two clone sets.

All subcultures derived from the inactive clones lost vector

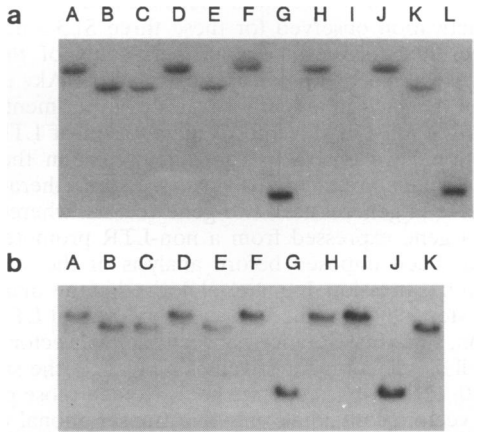


FIG. 5. Southern blot analysis of vector DNA in the subpopulations from Fig. 4. (a) Lanes: A to H, subpopulations at day 0 after selection; I, empty; J to L, original cell clone of S20, S55, S5. (b) Lanes: A to H, subpopulations after 64 days in cell culture without selection; I, J, and K, original cell clones of S20, S5, and S55. (a and b) Lanes A, D, F, and H, subpopulations of S20; lanes B, C, and E, subpopulations of S55; lane G, subpopulation of S5.

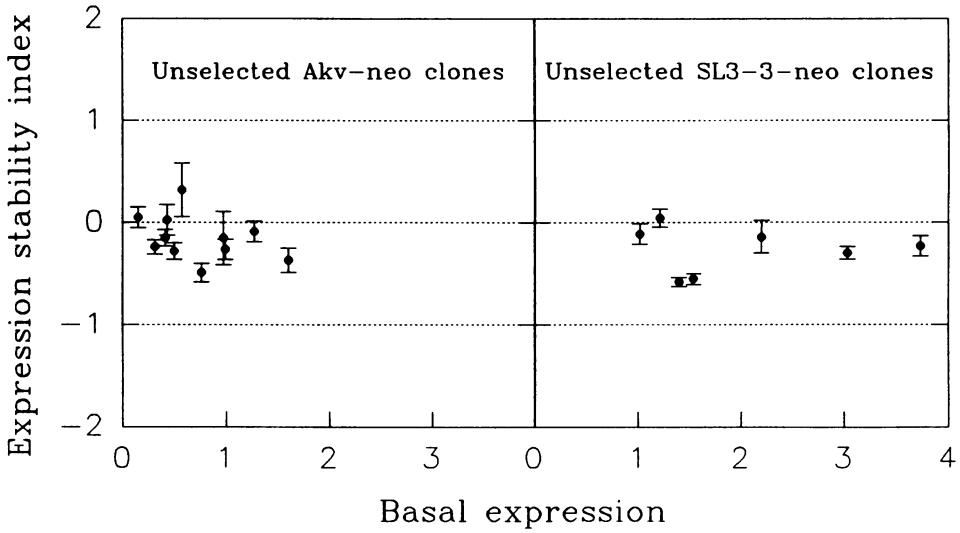


FIG. 6. Stability of expression in relation to expression levels. Regression coefficients are from analyses as exemplified in Fig. 3 as a function of basal transcriptional activity (NPT II) of the integrated vector in each Akv-neo and SL3-3-neo cell clone (26). The NPT II activities are given relative to that of a standard cell extract included in all sets of experiments (26). Clones were monitored for 144 days in cell culture, and two samples were taken for RNA extraction at each indicated time point (Fig. 3). Regression coefficients are given as percent per 100 days in cell culture. Error bars mark the 95% significance level.

expression with more rapid kinetics than those presented in Fig. 6. The regression coefficient, calculated as in Fig. 3, for the S5 subpopulation shows a higher negative value, −1.1 (SD = 0.14) than any of the subclones in Fig. 3, whereas regression coefficients cannot be calculated for the S20 and S55 subpopulations since they show no linear correlation between relative *neo* mRNA levels and days in cell culture. Although the activation treatment of these cultures might affect the kinetics of inactivation, it seems reasonable that an inherent increased susceptibility to inactivation made these three proviruses transcriptionally inactive at the initial isolation of cell clones. The expression levels of the subcultures after G418 selection fall within the range observed for the SL3-3-neo proviruses depicted in Fig. 6, in support of the notion that rapid inactivation is not limited to proviruses of a specific expression level. The rapid inactivation observed for these three SL3-3-neo proviruses does not seem to be a property only of the SL3-3 enhancer since inactive proviruses carrying the Akv enhancer have been detected in an earlier series of experiments (25).

Previous studies of the rate of inactivation of LTR-driven transcription have emerged from an interest in the use of murine leukemia virus-derived vectors in gene therapy. Such studies have in general used two-gene vectors, whereby selection for a gene expressed from a non-LTR promoter of the vector has been imposed before analysis of the stability of LTR-driven expression. In material derived in this manner, the selection step may introduce a bias in the levels of LTR-driven expression, since interference between the two vector promoters as well as selection cutoff values may affect the sample of clones (10, 11). In this study, we have avoided those problems by using vectors containing only one transcriptional unit in a set of cell clones initially isolated without selection for vector expression.

Although our results exclude a simple general relationship between the level and stability of provirus expression, the exact determinants of its susceptibility to inactivation remain unknown. The results are compatible with a single-step transition from the full expression level characteristic of a given clone to complete shutoff, with a frequency of inactivation also characteristic of a given integrated provirus (16, 18). Additional studies using single-cell expression analysis would be required to test this possibility further.

Long-term suspension cultures as used in this study will allow the selective outgrowth of cells having only a minor growth advantage. Differential effects of provirus expression on cell growth among the cell clones may therefore contribute to the observed differences in expression shutdown by an indirect mechanism. A possible effect of proviral expression level as such on cell growth cannot alone explain our results since clones with high vector expression levels are not particularly prone to loss of provirus expression upon cultivation. Alternatively, an effect on cell growth may depend on the proviral integration site. Studies of the interference between specific proviruses and their flanking host DNA will be required to test this possibility further.

From a study of the expression of Rous sarcoma virus proviruses in rat cells, a relationship between level and stability of expression was proposed; i.e., in contrast to the pattern in chick cells, the majority of Rous sarcoma virus proviruses are transcriptionally inactive in rat cells, in which regulatory proteins supporting LTR enhancer activity may be less prevalent (12, 19). If any such correlation between level and stability of expression exists for the murine proviruses used here, it does not operate in the range of expression levels available for study. It must also be stressed that the ability of the Src protein product of Rous sarcoma virus to influence proviral transcription via a positive feedback loop seems unparalleled for the murine vector proviruses studied here. The results and material presented here may be useful for future studies of factors affecting transcriptional inactivation of retroviral proviruses.

Furthermore, with the promising development of bicistronic retroviral vectors (4, 5, 21, 23), single-transcription-unit vectors as those used in the present study may be directly relevant as tools for gene therapy. Our results point to the use of vectors carrying a weak transcriptional LTR enhancer that is less harmful to the host cell in terms of insertional activation (20).

Although reduced in their ability to drive transient expression, vectors with a weak transcriptional enhancer may, when integrated, support as strong expression as a vector carrying a stronger LTR enhancer (Fig. 6) (26). As shown here, a reduced strength of the LTR enhancer does not make a vector provirus more susceptible to transcriptional inactivation.

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