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Different Relative Expression from Two Murine Leukemia Virus Long Terminal Repeats in Unintegrated Transfected DNA and in Integrated Retroviral Vector Proviruses

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Results of transient-expression studies have suggested a correlation between tissue-specific pathogenicity of murine leukemia viruses and the relative transcriptional activities of their long terminal repeats in various cell types. To test whether transient-expression ratios are representative of those of integrated proviruses, we developed a system for generation of retroviral transmission vectors differing only in U3. Vectors with the long terminal repeats of leukemogenic SL3-3 and nonleukemogenic Akv viruses were used for infection of a lymphoid cell line. We then compared expression in infected cells with transient expression after DNA transfection. In contrast to a high SL3-3/Akv reporter gene expression ratio in the transient assays, the ratio in stably infected populations was low. Sets of random cell clones from the two infected populations showed wide variation, with a mean value ratio identical to the population ratio but a considerably higher ratio between lowest values. We suggest that the lower expression levels, like transient expression, reflect inherent enhancer strength and that the higher levels represent chromosomal influence. The different pathogenicity, despite the moderate difference in average expression, may then relate to a different capacity for insertional oncogene activation owing to the different inherent enhancer strengths revealed by the transient-expression assays and the least active proviruses.

For a number of replication-competent murine leukemia viruses (MuLVs), differences in tumor-forming potential and tissue specificity of tumor formation have been shown to be determined by nucleotide sequences in the U3 region of the long terminal repeat (LTR) (5, 6, 9, 10, 16, 17, 21, 22, 34, 45, 46).

A correlation has been suggested between the transcriptional strength of a U3 region in a given cell type and the potential of the virus for tumor formation in the corresponding tissue (2–4, 23, 37, 41, 42, 48). This hypothesis was based on transient expression of reporter genes under the control of various MuLV LTRs. In each cell type, higher levels of expression were found from LTRs of viruses that cause tumors of these cells than from those of viruses that do not.

The investigators proposed the following explanations (4, 37): (i) a high transcription rate is accompanied by high replication rate, leading to efficient spread of the virus in the target tissue; (ii) high transcriptional activity leads to high expression of a viral gene product involved in tumor formation; (iii) transcription rates reflect cell-specific enhancer strengths, the stronger enhancers being more likely to activate neighboring cellular proto-oncogenes.

These experiments measured the transcriptional activity of unintegrated vector DNA after transfection. With this approach, obscuring influences of cellular flanking sequences are avoided. However, since a retrovirus exerts its influence upon the cell while integrated as a provirus, effects of flanking sequences upon its expression must be taken into account when possible relations between transcription and tumorigenicity are considered.

To evaluate the influence of chromosomal environment upon proviral gene expression, we constructed an Akv MuLV-based transmission vector permitting replacement of the U3 region with that of most other MuLVs. We then determined the transcriptional activity ratio of LTRs from highly leukemogenic SL3-3 (21, 43) and non-leukemogenic Akv (43, 44) in populations of the lymphoid cell line L691 (26) stably infected with retroviral vectors and compared this ratio with the ratio obtained in transient-expression assays after DNA transfection.

We report that although the different pathogenic properties are accompanied by a large difference in transient expression in L691 cells, in agreement with previous results (2, 4, 37), this difference is dramatically reduced in the stably infected populations.

To study the expression of individual proviruses, we isolated cell clones from both populations and determined the distribution of expression levels in the two clone series. On the basis of the clonal distributions, we propose a model which can account for the discrepancy between the results obtained by the two methods of gene transfer and discuss the implications for the proposed mechanisms of tumor formation.

MATERIALS AND METHODS

Plasmids. The *neo* transmission vector plasmid pT-523 (pL-psi-PLneo) has been described earlier (20). For the multistep replacement in the downstream Akv LTR of the *Pst*I-*Kpn*I fragment with that of another retrovirus, see Fig. 1. The plasmid pGemH, lacking cleavage sites for *Pst*I, *Kpn*I, *Nru*I, and *Hin*cII, is derived from pGem1 and pGem2 (Promega Biotec). The p(tvSL3-3-neo) vector was generated by using the *Pst*I-*Kpn*I fragment from a plasmid carrying the SL3-3 provirus (kindly provided by J. Lenz). The p(tvAkv-neo) vector was regenerated from the pT590 and pT606 plasmids (see Fig. 2). The expression vector pAkv-cat has been described previously (29). The plasmid pSL3-3-cat was constructed by *Pst*I-*Kpn*I fragment replacement (see Fig. 2).

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Recombinant DNA work was carried out by standard procedures (24). The host bacterial strain was *Escherichia coli* K-803 (47).

Cell culture. psi-2 (25) and NIH 3T3 cells were grown in Dulbecco modified Eagle medium–10% calf serum. L691 is a T-lymphoid cell line derived from a radiation-induced thymoma of a C57L mouse (26). L691 cells were grown in suspension in RPMI 1640 medium–10% calf serum and cloned in RPMI 1640 medium–20% fetal calf serum. G418 was obtained from Sigma Chemical Co., St. Louis, Mo. (483 μg of geneticin base per mg).

Transfection and chloramphenicol acetyltransferase assay. psi-2 cells (25) were transfected by the calcium phosphate precipitation method (13) with the transfection cocktail of Chu and Sharp (7). L691 cells were transfected by the DEAE-dextran method of Sompayrac and Danna (38) as described by Queen and Baltimore (31), and 36 h later cell extracts were prepared by four cycles of freeze-thawing. The expression of chloramphenicol acetyltransferase was determined as described by Gorman (12).

Infection with recombinant retroviruses. Plasmids p(tvAkv neo) and p(tvSL3-3-neo) were transfected into psi-2 cells, and G418-resistant colonies were isolated. The production of virus particles containing vector RNA was assayed by titer determination on NIH 3T3 cells, and high-titer psi-2 colonies were chosen for further experiments. psi-2 producer cells were plated at a density of 2.5×10^5 cells per T25 culture flask in Dulbecco modified Eagle medium. On the following day, 2.5×10^5 L691 recipient cells were added to each flask, and Polybrene (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was added to 8 μg/ml. After 3 days the infected L691 populations were transferred to new flasks and diluted with RPMI 1640 medium. Single-cell clones were established 48 h later by endpoint dilution. The cloning efficiency was determined as the number of wells giving rise to viable clones as a percentage of the number of wells originally containing a single cell as monitored microscopically.

NPT II assay. Cells were harvested at a concentration of 5×10^5 to 1.5×10^6 cells per ml. A 1.5-ml volume of cell culture was centrifuged, washed twice with phosphate-buffered saline and suspended in 100 μl of 0.135 M Tris hydrochloride (pH 6.8)–20% glycerol–4 mM dithiothreitol. Crude extracts were prepared by disrupting the cells by four rounds of freeze-thawing. Cell debris were removed by centrifugation, and the supernatant was stored at −20°C. Assays for neomycin phosphotransferase (NPT) II activity were performed essentially as described previously (30), with the following modifications: 10 μl of the cell extract (1 to 8 μg of protein) was mixed with 200 μl of 67 mM Tris hydrochloride (pH 7.1), 42 mM $MgCl_2$, 400 mM NH_4Cl, 40 μg of kanamycin sulfate per ml, and 2.5 to 10 μCi of [γ-^{32}P]ATP (carrier free, crude; ICN Radiochemicals) per ml. The mixture was incubated for 135 min at 27°C before filtration through a nitrocellulose-phosphocellulose filter sandwich as described by Platt and Yang (30). Spots containing kanamycin phosphate were cut from the phosphocellulose filter, and radioactivity was measured by scintillation counting. The protein content of the samples was measured directly on the nitrocellulose filters after staining with Amido Schwarz. The completely wetted filter was layered between two glass plates, and optical densities of the protein spots were measured by using an enzyme-linked immunosorbent assay reader at 620 nm with unstained nitrocellulose as a reference and related to a standard curve of known protein concentrations.

NPT II activity is given as specific activity (cpm per microgram of protein) or specific activity relative to that of a standard cell extract arbitrarily set to a value of 1, as specified in the figure legends. All NPT II determination experiments were performed at least three times.

RNA analysis. Total cellular RNA was isolated from 10^6 cells by the hot acid-phenol-sodium dodecyl sulfate procedure as previously described (20). RNase protection analysis (28) was carried out as described previously (20), except for a change of hybridization temperature to 55°C.

Southern blot analysis. Southern blotting (39), nick translation (33), and hybridizations were carried out as recommended by Meinkoth and Wahl (27).

Polymerase chain reaction. The nucleotide sequences of upstream proviral LTRs were determined after amplification with the polymerase chain reaction (35, 36) and oligonucleotide primers located in the *neo* gene (nucleotides 1659 to 1686, 5′-GGCGCCCCTGCGCTGACAGCCGGAACAC-3′) (1) and upstream in the U3 region (nucleotides 14 to 41, 5′-TTCATAAGGCTTAGCCAGCTAACTGCAG-3′) (44). Taq polymerase and the protocol recommended (Perkin-Elmer-Cetus) were used, except that the annealing temperature was raised to 62°C. Denaturation and annealing times were 1.2 min, and the extension time was set to 5 min to amplify fragments of 1,375 nucleotides. The polymerase chain reaction products were purified, and sequencing was performed with the Sequenase kit provided by U.S. Biochemicals Corp., Cleveland, Ohio.

RESULTS

Generation of transmission vectors with variant U3 regions. For the analysis of cell-specific expression of integrated proviruses, we chose to use replication-defective viruses, carrying the bacterial NPT II gene, *neo* (1), in place of the viral genes. With this approach, we avoid superinfection of cells with low provirus expression and facilitate Southern blot analysis of provirus copy numbers.

We developed a system for generation of retroviral transmission vectors differing only in U3. The principle of multistep U3 replacement is shown in Fig. 1. Starting from the Akv-based *neo*-transmission vector plasmid pT-523 (20), the downstream Akv U3 region is exchanged with the desired U3 region. The resulting plasmid is transfected into the psi-2 packaging cell line, and the vector RNA-containing viral particles produced are used for infection of other cells. The integrated vector DNA in these cells will have the substituted LTR at both ends of the provirus.

The presence of the *neo* gene permits G418 selection (8, 40) of *neo*-positive, high-titer colonies of the packaging cell line, and its expression in infected cells can be measured rapidly by using crude cell extracts (14, 30, 32). It also permits studies employing selection for reporter gene expression in infected cells (K. Paludan, M. Duch, P. Jørgensen, N. O. Kjeldgaard, and F. S. Pedersen, Gene, in press).

The SL3-3 vector plasmid p(tvSL3-3-neo) was generated by the above substitution procedure, with the *PstI-KpnI* fragment from a plasmid carrying an SL-3 provirus (kindly provided by J. Lenz) (Fig. 2). The structure of the replaced U3 region was confirmed by sequence analysis. Since R, U5, and the U3 sequences upstream of the *PstI* site are identical in Akv and SL3-3 (43), the replaced LTR of p(tvSL3-3-neo) is identical to the LTR of the SL3-3 provirus. Akv vector plasmid p(tvAkv-neo) was generated from unsubstituted pT590 and pT606.

Transient expression in transfected populations. For the transfection experiments, two plasmid vectors, pAkv-cat

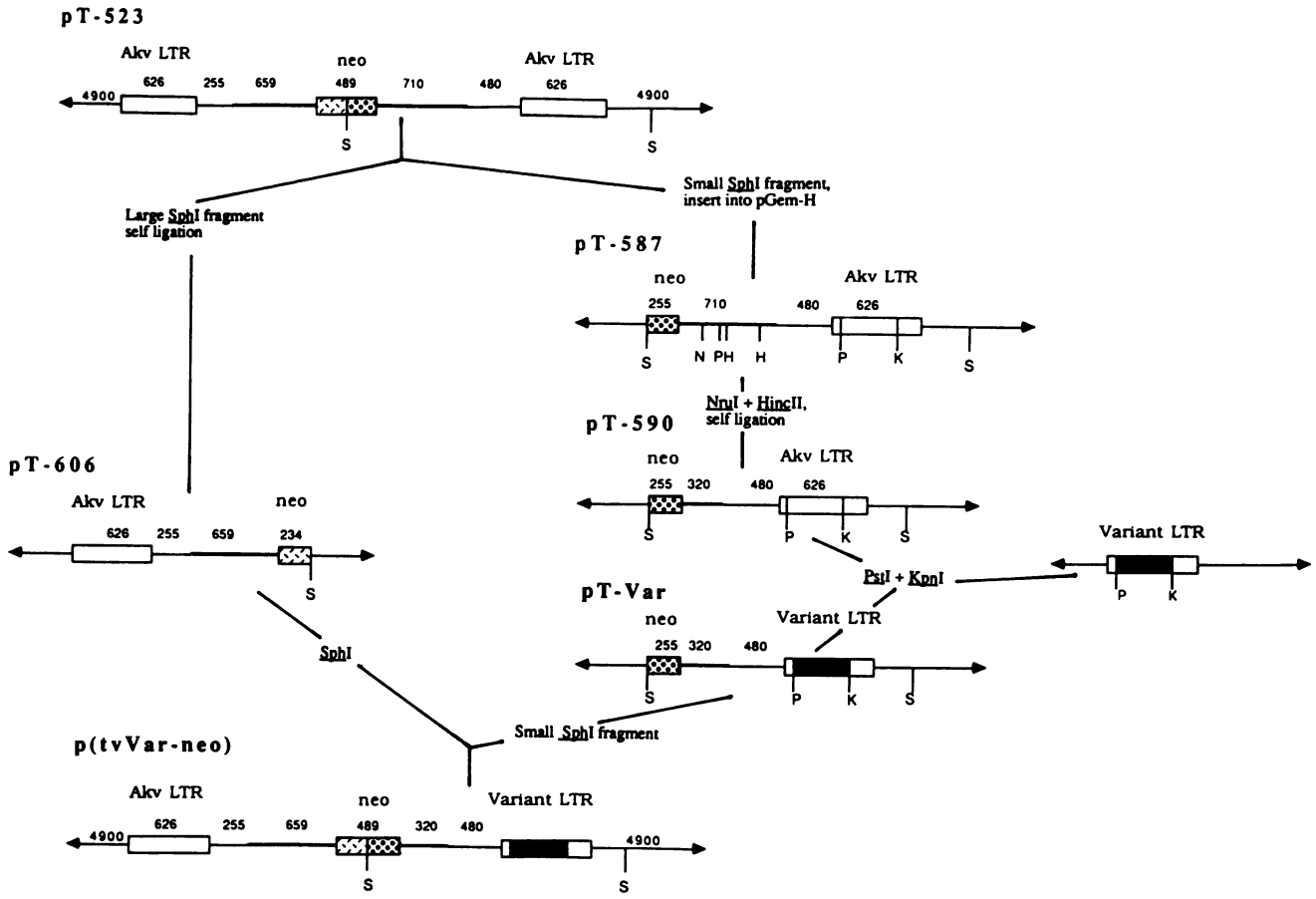

FIG. 1. Construction scheme for U3 region replacement in transmission vector plasmids. For U3 replacement, the *Kpn*I site in R and the *Pst*I site in the upstream part of U3 were chosen, since these are present in most MuLVs. To circumvent the problem of multiple *Pst*I sites in pT-523, we subcloned the smaller *Sph*I fragment of pT-523 and deleted the *Pst*I site of a nonessential region. Plasmid pT-523 was cleaved with *Sph*I. The larger *Sph*I fragment was self-ligated to form plasmid pT-606, and the smaller *Sph*I fragment was subcloned in the pGem-H plasmid to yield the plasmid pT-587. This plasmid was cleaved with *Nru*I and *Hinc*II and self-ligated to give the plasmid pT-590, which has a single *Pst*I site. By cutting with *Pst*I and *Kpn*I, most of the U3 region and part of the R region of the Akv LTR will be removed and can be replaced by the corresponding *Pst*I-*Kpn*I fragment from a suitable LTR region. From the resulting plasmid, pT-Var, the smaller *Sph*I fragment is subcloned into the *Sph*I site of pT-606, whereby an intact *neo* gene is reestablished in the transmission vector plasmid p(tvVar-neo). These plasmids express a functional *neo* gene and render their host bacteria resistant to kanamycin, ensuring insertion of the fragment in the correct orientation. Abbreviations: H, *Hinc*II; K, *Kpn*I; N, *Nru*I; P, *Pst*I; S, *Sph*I. Heavy lines surrounding the *neo* gene are Tn*5* sequences.

and pSL3-3-cat, were used, in which the chloramphenicol acetyltransferase gene is under the control of Akv and SL3-3 LTRs, respectively (Fig. 2). The lymphoid cell line L691 was chosen because it has been shown previously to discriminate between the two LTRs in transient-expression assays (2, 4, 37). L691 was transfected with plasmids pAkv-cat and pSL3-3-cat by the DEAE-dextran method. After 48 h, cell extracts were prepared and chloramphenicol acetyltransferase enzymatic activity was measured (Table 1). The population transfected with pSL3-3-cat shows a chloramphenicol acetyltransferase activity 20 to 30 times that of the pAkvcat-transfected population, confirming the large difference in transient expression found by others (2, 4, 37).

Expression in stably infected populations. L691 recipient cells were infected by a 3-day cocultivation with psi-2 cells producing tvAkv-neo or tvSL3-3-neo. No helper viruses could be detected in the infected populations by cocultivation with NIH 3T3 cells. One week after infection, cell extracts were prepared and NPT II enzymatic activity measured. Infection efficiencies were calculated from the number of NPT II-negative clones among clones isolated from each infected population (see below), and the population activities were corrected for infection efficiency (Table 1). In the infected populations, the SL3-3/Akv expression ratio is only 2.

Isolation of cell clones and identification of vector-positive clones. Since cell-to-cell variation is known to occur between identical proviruses integrated at different sites (11, 18, 19), the average values obtained by population measurements might reflect wide cell-to-cell variation. We therefore wanted to analyze the distribution of NPT II activities in the two populations. Cell clones were isolated by endpoint dilution in nonselective medium. We obtained 35 clones from the tvAkv-neo-infected population (L691/Akv) (cloning efficiency, 77%) and 75 clones from the tvSL3-3-neo-infected population (L691/SL3-3) (cloning efficiency, 83%). By using Southern blot hybridization of DNA from all clones to a probe corresponding to the coding region of the *neo* gene, we identified 16 and 13 *neo*-positive clones among the 35 L691/Akv and the 75 L691/SL3-3 clones, respectively. We did not

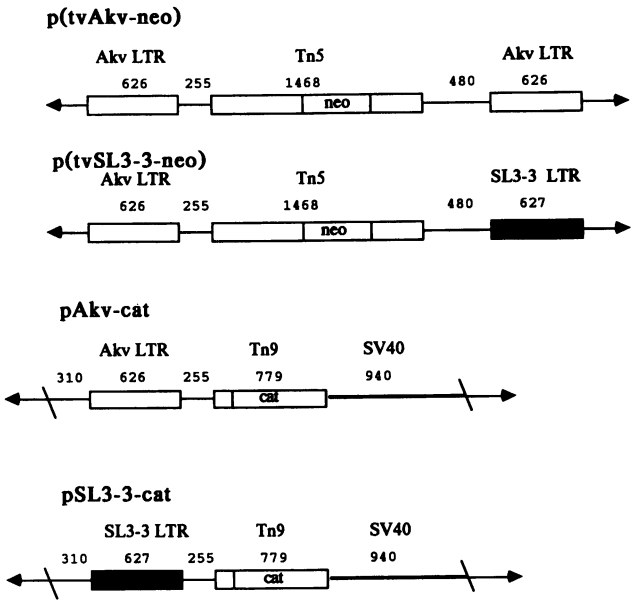

FIG. 2. Genetic map of vectors used for infection and transfection experiments. The transmission vector plasmids [p(tvAkv-neo) and p(tvSL3-3-neo)] carry a 5′ Akv LTR with 255 base pairs of flanking 5′ untranslated sequences of Akv MuLV followed by a Tn5 fragment with the open reading frame of *neo* (indicated) and 480 base pairs of Akv MuLV sequences. As indicated, the plasmids differ only in the 3′ LTR sequences. The arrows denote bacterial vector plasmid sequences. The vector plasmids used for DNA-mediated gene transfer (pAkv-cat and pSL3-3-cat) carry a 5′ LTR of either Akv or SL3-3 surrounded by 255 base pairs of 5′ untranslated sequences and 310 base pairs of 3′ sequences from Akv MuLV. The Tn*9* fragment contains the open reading frame for the chloramphenicol acetyltransferase gene (indicated). The simian virus 40 sequences carrying a polyadenylation site were derived from pSV2cat. The arrows denote bacterial vector plasmid sequences.

use selection for expression of the *neo* gene to identify infected cells prior to cloning, because we did not want to select against cells with low levels of expression.

Reporter gene expression in infected clones. For each clone identified as *neo* vector positive by Southern blot analysis, NPT II enzymatic activity was measured. The results for the NPT II active clones are shown in Fig. 3. The activities show a clonal variation ranging from 0.10 to 2.55 relative units for the L691/Akv series and from 0.88 to 3.73 relative units for the L691/SL3-3 series. When compared in a Mann-Whitney U test, the two distributions show a statistically significant difference at the 0.5% level, but they overlap and show a ratio of mean values of only 2, in agreement with the population measurements (Table 1).

TABLE 1. Reporter gene expression in transfected and infected populations

Gene transfer method	Gene expression for:		SL3-3/Akv expression ratio
	SL3-3	Akv	
Transfection[a]			
Expt 1	10.2×10^{-3}	3.2×10^{-4}	32
Expt 2	4.6×10^{-3}	2.1×10^{-4}	22
Infection			
Populations[b]	629	312	2.0
Clones[c]	1.78	0.88	2.0

[a] Expression is given as CAT activity (percent conversion per minute per microgram of protein). Each number represents the average of three independent results, none of which varied by more than 20% of the average value.

[b] Expression is given as NPT II activity (counts per minute per microgram of protein), corrected for NPT II-positive infection efficiency, μ, calculated from the number of NPT II-negative clones in each clone series by using the *P*(0) term of the Poisson distribution. Each number represents the average of four independent results, none of which varied by more than 20% of that value.

[c] Average NPT II activity of NPT II-positive clones from the infected populations (Fig. 3). NPT II activity is given as counts per minute per microgram of protein relative to a standard cell extract common to all experiments with clone extracts.

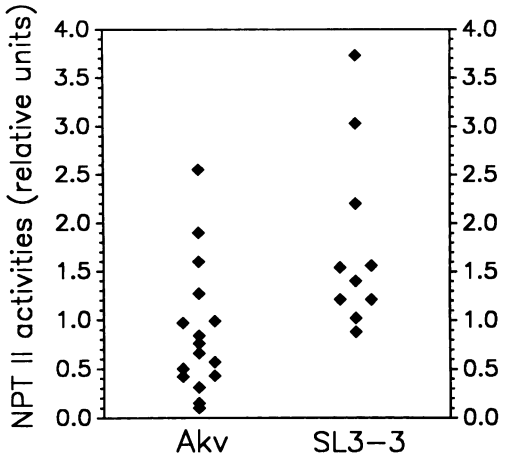

FIG. 3. NPT II activity distributions of vector-positive clones from the infected populations. Each point represents the activity of one clone and is the average of three independent results. The mean deviation from the average was 15% of that value. NPT II activity is given as counts per minute per microgram of protein, relative to a standard cell extract common to all experiments. Three L691/SL3-3 clones were completely inactive and are not represented in the diagram (see Results). Most clones have one integrated vector copy. For those with more than one copy, the NPT II activities are as follows: three copies, 1.90 units (Akv); two copies, 0.10 (Akv), 0.66 (Akv), 0.84 (Akv), 1.21 (SL3-3), 1.56 (SL3-3), and 2.55 (Akv) units. Sample mean, standard deviation, and standard error of the mean for the two samples are as follows: 0.88, 0.67, and 0.17, respectively, for Akv, and 1.78, 0.93, and 0.30, respectively, for SL3-3.

In contrast, the ratio of the lowest values of the SL3-3 series to the lowest values of the Akv series is higher, 7 to 9, and in closer agreement with the high SL3-3/Akv activity ratio of the transient-transfection experiments. Both distributions show a positive skewness, with an accumulation of clones toward the lower values.

We believe that the NPT II activity distributions of the clones are representative of the activity distributions of their respective populations, since cloning efficiencies were high for both populations and since NPT II activities of the two populations, when corrected for infection efficiencies, showed the same ratio. That the cloning procedure has not specifically removed high SL3-3 values is supported by the fact that the standard deviation of the L691/SL3-3 distribution is not lower than that of the L691/Akv distribution (Fig. 3). No correlation was observed between NPT II activities and growth rates in nonselective medium (data not shown).

Three vector-positive clones from the L691/SL3-3 series showed activities indistinguishable from that of uninfected L691, several hundred times lower than those of the rest of the L691/SL3-3 clones and thus completely outside the distribution. Such total lack of expression of the reporter gene has been observed in earlier experiments with this vector, with tvAkv-neo, and with the transmission vector

TABLE 2. Poisson distribution of *neo*-vector DNA copy numbers among clones

Parameter	Value of parameter for:	
	L691/SL3-3	L691/Akv
No. of clones isolated	75	35
No. of vector-negative clones	62	19
Avg infection efficiency, μ[a]	0.19	0.61
Fraction[b]		
Vector negative	0.83	0.54
One copy	0.16	0.33
Two copies	0.015	0.10
Three copies	0.001	0.02
No. of clones, calculated[c]		
One copy	12.0	11.6
Two copies	1.1	3.5
Three copies	0.1	0.7
No. of clones, observed		
One copy	11	11
Two copies	2	4
Three copies	0	1

[a] The average number of infectious events was calculated from the number of vector-negative cell clones by using the $P(0)$ term of the Poisson distribution, $P(0) = e^{-\mu}$.
[b] The fractions were calculated from the Poisson distribution.
[c] Calculated by using the fractions above.

L-psi-PLneo (20). We believe that vector technical problems (mutations or deletions) have caused this phenomenon. Alternatively, since we have found no mutations or deletions on preliminary sequence analysis of proviral LTR DNA from these clones, the proviruses may be integrated in chromosomal regions that abolish transcriptional activity. In either case, the phenomenon is irrelevant to the question of cell-specific LTR activity, and the three clones are not included in Fig. 3.

Vector provirus copy numbers of infected clones. For Southern blot analysis, DNA from all clones was digested with *Hin*dIII. Since there is only one *Hin*dIII site in the vector sequence, located at the 5′ border of the Tn*5* sequence, the sizes of restriction fragments hybridizing to the *neo* probe depend on the locations of cellular *Hin*dIII sites. Thus, proviruses inserted at different chromosomal locations give hybridization bands of different sizes, permitting determination of the number of inserted vector proviruses of each clone.

Infection with retroviral vectors usually results in only one or a few integrated copies, a situation that may be advantageous for studies of normal gene regulation, as opposed to the high copy numbers (15) in cells transfected with vector DNA. In our case the copy number is indeed low, with 1 as the most frequent number and 3 as the highest (see the legend to Fig. 3). From the number of noninfected clones in each clone set, we calculated the average number of infectious events per cell, μ, by using the 0 class term of the Poisson distribution, $P(0) = e^{-\mu}$, (Table 2). With this average value, we again used the Poisson distribution to calculate the expected number of cells having been subjected to one or more infections. The calculated values and the observed number of clones containing one, two, or three vector DNA copies were in good agreement (Table 2). This supports the notion that infectious events occur at random and that an infectious event has an equal chance of resulting in insertion of a vector provirus, whether it is the first, second, or third.

RNA analysis. To test whether readthrough from flanking sequences was a major contributor to clonal variation in NPT II expression, we analyzed a subset of four L691/Akv and four L691/SL3-3 clones of different activities by RNase protection with a radioactive cRNA probe protecting part of U3, R, U5, and a small part of the 5′ untranslated region (20). Normal initiation and polyadenylation will result in bands of 213 and 299 nucleotides, respectively. For all clones tested, these two bands occurred with about equal intensities, and no additional major bands were found (data not shown).

Sequence analysis. To test whether the SL3-3 vector provirus contained the SL3-3 5′ LTR expected to be generated during reverse transcription in infected cells, the 5′ LTR of the single vector provirus of five L691/SL3-3 clones was amplified by the polymerase chain reaction method, with oligonucleotide primers located in the *neo* gene and in the upstream part of U3, immediately 3′ to the inverted repeat. Sequence analysis of the amplified DNA confirmed the presence of the SL3-3 U3 region originally inserted into the 3′ LTR of the vector plasmid. Preliminary analysis of polymerase chain reaction-amplified DNA from eight L691/Akv clones has revealed no rearrangements or mutations in the U3 region.

DISCUSSION

The cell-specific pathogenicity of MuLVs has been correlated with the relative transcriptional activities of their LTRs in different cell types (2–4, 23, 37, 41, 42, 48) as measured from unintegrated vector DNA after transfection. In this study we tested whether transcription ratios obtained in this way reflect the ratios of transcription from integrated proviruses.

Using transfected cells selected for stable *neo* gene expression, Yoshimura and Chaffin (48) observed a reduced expression ratio between MCF-13 and Akv LTRs relative to transient-expression ratios. In our experience, however, comparison of G418-selected populations is not informative of relative expression levels, since standard selection procedures eliminate cells of low expression, thereby preferentially raising the average expression of the population with the lowest expression levels, in our case L691/Akv (Paludan et al., in press). The efficiency of retroviral vector-mediated gene transfer enabled us to omit a selection step and thus avoid biased selection as a source of error. In addition, this gene transfer method gives low copy numbers and well-defined transcription units, a situation more relevant to the discussion of viral pathogenesis.

For the study of proviral gene expression, we constructed a system for easy generation of retroviral *neo* transmission vectors differing only in U3. Since it will accommodate the U3 regions of most MuLVs and since the vector RNA can be packaged in the widely used psi-2 packaging cell line, this system should be useful for comparative analyses of cell-specific enhancer activity. In the work presented here, it was used for construction of vectors with the LTRs of MuLVs Akv and SL3-3. These vectors were then used for infection of the lymphoid cell line L691.

The SL3-3 vector plasmid had the SL3-3 LTR only in the 3′ end, and the validity of our results depends on the correct transfer to the 5′ end during reverse transcription in infected cells. Sequence analysis of polymerase chain reaction-amplified 5′ proviral LTR DNA of five L691/SL3-3 clones confirmed the presence of the correct SL3-3 sequence. For some vector-positive clones from each population, vector RNA was analyzed. In all cases tested, the standard vector

transcription unit was active and we observed no evidence of additional RNA species. We therefore believe that NPT II activities in the infected populations reflect the activities of the SL3-3 and Akv LTRs.

We compared expression in the two infected populations with transient expression in L691 populations transfected with plasmid vectors with the same two LTRs. In the transient-expression assays we found SL3-3/Akv expression ratios of 20 to 30, confirming that the relative activities in a lymphoid cell line of the two LTRs correlate well, when measured by this method, with the fact that SL3-3 is highly leukemogenic whereas Akv is not. In a parallel experiment, Celander and Haseltine (4) and Short et al. (37) found ratios of about 6 and 17, respectively, in this cell line. In contrast, when we measured reporter gene expression in populations infected with the retroviral vectors, we found a SL3-3/Akv expression ratio of only 2.

To analyze the cellular distributions behind the average values obtained by population measurements, we determined NPT II activity for all vector-positive clones in representative clone samples from the two infected populations. Both samples showed a wide variation, with the L691/Akv sample spanning a factor of 25 and the L691/SL3-3 sample spanning a factor of 4. The larger variation in the L691/Akv sample is mostly due to the presence of low expression levels not found in L691/SL3-3. The two distributions overlap and show a ratio between mean values of 2, corresponding to the ratio between the populations.

If the reporter gene expression of our retroviral vector proviruses is representative of wild-type virus transcription rates, we must therefore assume that the transcriptional difference between SL3-3 and Akv during a natural infection of lymphoid cells is, on the average, not very pronounced. Furthermore, the higher values in our L691/Akv clone expression level distribution suggest that Akv may occasionally be expressed as strongly as SL3-3. This finding is compatible with the observation of Rosen et al. (34) that on rare occasions Akv can infect and multiply in various tissues as efficiently as SL3-3.

For these reasons, it may be difficult to accept virus expression level as the sole explanation of pathogenic phenotype. Also, the moderate difference between the proviral expression levels seems to conflict with the very strict differences between the viruses in transient-expression levels in lymphoid cells as well as in pathogenic properties.

Both clone samples showed a clustering of clones toward the lower expression levels. When the lowest values obtained for each series are considered, a ratio is observed that is considerably larger than the mean value ratio; it therefore corresponds better to the results of the transient-expression assays. It is therefore tempting to associate these lower values both with the transient-expression values and with relative pathogenicity. If transient-expression assays reveal the basic strength of an enhancer in the cellular environment of diffusible factors (2, 42), the lowest expression levels observed among our NPT II-positive clones may then represent proviruses integrated in chromosomal regions that do not exert a very strong influence upon the transcription of the proviral genes and hence do not affect the inherent basic values very much, whereas the higher values may represent positive chromosomal influence. The ability of a proviral enhancer to activate neighboring proto-oncogenes may be assumed to depend on the inherent strength of the enhancer, not on the activity to which it can be stimulated by the surroundings. Insertional activation or stimulation of a silent or weakly expressed cellular proto-oncogene may be supposed to occur in chromosomal regions of weak transcriptional activity; these are the regions in which the unmodified proviral enhancer strengths are revealed. Thus, our model discriminates between the proposed mechanisms for tumor formation by replication-competent murine retroviruses in favor of the insertional activation hypothesis.

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LITERATURE CITED

1. **Beck, E., E. A. Ludwig, R. Auerswald, B. Reiss, and H. Schaller.** 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. Gene **19:** 327–336.
2. **Boral, L. B., S. A. Okenquist, and J. Lenz.** 1989. Identification of the SL3-3 virus enhancer core as a T lymphoma cell specific element. J. Virol. **63:**76–84.
3. **Bösze, Z., H.-J. Thiesen, and P. Charnay.** 1986. A transcriptional enhancer with specificity for erythroid cells is located in the long terminal repeat of the Friend murine leukemia virus. EMBO J. **5:**1615–1623.
4. **Celander, D., and W. A. Haseltine.** 1984. Tissue-specific transcription preference as a determinant of cell tropism and leukaemogenic potential of murine retroviruses. Nature (London) **312:**159–162.
5. **Chatis, P. A., C. A. Holland, J. W. Hartley, W. P. Rowe, and N. Hopkins.** 1983. Role for the 3′ end of the genome in determining disease specificity of Friend and Moloney murine leukemia viruses. Proc. Natl. Acad. Sci. USA **80:**4408–4411.
6. **Chatis, P. A., C. A. Holland, J. E. Silver, T. N. Frederickson, N. Hopkins, and J. W. Hartley.** 1984. A 3′ end fragment encompassing the transcriptional enhancers of nondefective Friend virus confers erythroleukemogenicity on Moloney leukemia virus. J. Virol. **52:**248–254.
7. **Chu, G., and P. A. Sharp.** 1981. SV40 DNA transfection of cells in suspension: analysis of the efficiency of transcription and translation of T-antigen. Gene **13:**197–202.
8. **Colbère-Garapin, F., F. Horodniceanu, P. Kourilsky, and A.-C. Garapin.** 1981. A new dominant hybrid selective marker for higher eukaryotic cells. J. Mol. Biol. **150:**1–14.
9. **DesGroseillers, L., and P. Jolicoeur.** 1984. The tandem direct repeats within the long terminal repeat of murine leukemia viruses are the primary determinant of their leukemogenic potential. J. Virol. **52:**945–952.
10. **DesGroseillers, L., E. Rassart, and P. Jolicoeur.** 1983. Thymotropism of murine leukemia virus is conferred by its long terminal repeat. Proc. Natl. Acad. Sci. USA **80:**4203–4207.
11. **Feinstein, S. C., S. R. Ross, and K. R. Yamamoto.** 1982. Chromosomal position effects determine transcriptional potential of integrated mammary tumor virus DNA. J. Mol. Biol. **156:**549–565.
12. **Gorman, C.** 1985. High efficiency gene transfer into mammalian cells, p. 143–190. *In* D. M. Glover (ed.), DNA cloning, vol. II. IRL Press, Oxford.
13. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
14. **Haas, M. J., and J. E. Dowding.** 1975. Aminoglycoside-modifying enzymes. Methods Enzymol. **43:**611–627.
15. **Hwang, L.-H. S., and E. Gilboa.** 1984. Expression of genes introduced into cells by retroviral infection is more efficient than that of genes introduced into cells by DNA transfection. J. Virol. **50:**417–424.
16. **Ishimoto, A., A. Adachi, K. Sakai, and M. Matsuyama.** 1985. Long terminal repeat of Friend-MCF virus contains the se-

quence responsible for erythroid leukemia. Virology **141**:30–42.
17. **Ishimoto, A., M. Takimoto, A. Adachi, M. Kakuyama, S. Kato, K. Kakimi, K. Fukuoka, T. Ogiu, and M. Matsuyama.** 1987. Sequences responsible for erythroid and lymphoid leukemia in the long terminal repeats of Friend-mink cell focus forming and Moloney murine leukemia viruses. J. Virol. **61**:1861–1866.
18. **Jaenisch, R., D. Jähner, P. Nobis, I. Simon, J. Lohler, K. Harbers, and D. Grotkopp.** 1981. Chromosomal position and activation of retroviral genomes inserted into the germ line of mice. Cell **24**:519–529.
19. **Jähner, D., and R. Jaenisch.** 1980. Integration of Moloney leukemia virus into the germ line of mice: correlation between site of integration and virus activation. Nature (London) **287**: 456–458.
20. **Jensen, N. A., P. Jørgensen, N. O. Kjeldgaard, and F. S. Pedersen.** 1986. Mammalian expression-and-transmission vector derived from Akv murine leukemia virus. Gene **41**:59–65.
21. **Lenz, J., D. Celander, R. L. Crowther, R. Patarca, D. W. Perkins, and W. Haseltine.** 1984. Determination of the leukaemogenicity of a murine retrovirus by sequences within the long terminal repeat. Nature (London) **308**:467–480.
22. **Li, Y., E. Golemis, J. W. Hartley, and N. Hopkins.** 1987. Disease specificity of nondefective Friend and Moloney murine leukemia viruses is controlled by a small number of nucleotides. J. Virol. **61**:693–700.
23. **LoSardo, J. E., L. A. Cupelli, M. K. Short, J. W. Berman, and J. Lenz.** 1989. Differences in activity of murine retroviral long terminal repeats in cytotoxic T lymphocytes and T lymphoma cells. J. Virol. **63**:1087–1094.
24. **Maniatis, F., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Mann, R., R. C. Mulligan, and D. Baltimore.** 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell **33**:153–159.
26. **McGrath, M. S., E. Pillemer, D. Kooistra, and I. L. Weissmann.** 1980. The role of MuLV receptors on T-lymphoma cells in lymphoma cell proliferation, p. 157–184. *In* N. L. Warner (ed.), Contemporary topics in immunology. Plenum Publishing Corp., New York.
27. **Meinkoth, J., and G. Wahl.** 1984. Hybridization of nucleic acids immobilized on solid supports. Anal. Biochem. **138**:267–284.
28. **Melton, D. A., P. A. Krieg, M. R. Rebagliata, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmid containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
29. **Pedersen, F. S., M. Etzerodt, S. Lovmand, H. Y. Dai, A. J. Bækgaard, J. Sørensen, P. Jørgensen, N. O. Kjeldgaard, J. Schmidt, C. Leib-Mösch, A. Luz, and V. Erfle.** 1987. Transcriptional control and oncogenicity of murine leukemia viruses, p. 17–35. *In* N. O. Kjeldgaard and J. Forchhammer (ed.), Viral carcinogenesis. Alfred Benzon Symposium no. 24. Munksgaard, Copenhagen.
30. **Platt, S. G., and N.-S. Yang.** 1987. Dot assay for neomycin phosphotransferase activity in crude cell extracts. Anal. Biochem. **162**:529–535.
31. **Queen, C., and D. Baltimore.** 1983. Immunoglobulin gene transcription is activated by downstream sequence elements. Cell **33**:741–748.
32. **Reiss, B., R. Sprengel, H. Will, and H. Schaller.** 1984. A new sensitive method for qualitative and quantitative assay of neomycin phosphotransferase in crude cell extracts. Gene **30**: 211–218.
33. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
34. **Rosen, C. A., W. A. Haseltine, J. Lenz, R. Ruprecht, and M. W. Cloyd.** 1985. Tissue selectivity of murine leukemia virus infection is determined by long terminal repeat sequences. J. Virol. **55**:862–866.
35. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.
36. **Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H.-A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230**:1350–1354.
37. **Short, M. K., S. A. Okenquist, and J. Lenz.** 1987. Correlation of leukemogenic potential of murine retroviruses with transcriptional tissue preference of the viral long terminal repeats. J. Virol. **61**:1067–1072.
38. **Sompayrac, L. M., and K. J. Danna.** 1981. Efficient infection of monkey cells with DNA of simian virus 40. Proc. Natl. Acad. Sci. USA **78**:7575–7578.
39. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
40. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
41. **Thiesen, H.-J., Z. Bösze, L. Henry, and P. Charnay.** 1988. A DNA element responsible for the different tissue specificities of Friend and Moloney retroviral enhancers. J. Virol. **62**:614–618.
42. **Thornell, A., B. Hallberg, and T. Grundström.** 1988. Differential protein binding in lymphocytes to a sequence in the enhancer of the mouse retrovirus SL3-3. Mol. Cell. Biol. **8**:1625–1637.
43. **Van Beveren, C., J. Coffin, and S. Hughes.** 1985. Nucleotide sequences complemented with structural and functional analysis, p. 567–1148 (appendix B). *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, vol. 2. Supplements and appendixes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Van Beveren, C., E. Rands, S. K. Chattopadhyay, D. R. Lowy, and I. M. Verma.** 1982. Long terminal repeat of murine retroviral DNAs: sequence analysis, host-proviral junctions, and preintegration site. J. Virol. **41**:542–556.
45. **Villemur, R., E. Rassart, L. DesGrosseillers, and P. Jolicoeur.** 1983. Molecular cloning of viral DNA from leukemogenic Gross passage A murine leukemia virus and nucleotide sequence of its long terminal repeat. J. Virol. **45**:539–546.
46. **Vogt, M., C. Haggblom, S. Swift, and M. Haas.** 1985. Envelope gene and long terminal repeat determine the different biological properties of Rauscher, Friend, and Moloney mink cell focus-inducing viruses. J. Virol. **55**:184–192.
47. **Wood, W. B.** 1966. Host specificity of DNA produced by Escherichia coli: bacterial mutations affecting restriction and modification of DNA. J. Mol. Biol. **16**:118–133.
48. **Yoshimura, F. K., and K. Chaffin.** 1987. Different activities of viral enhancer elements before and after stable integration of transfected DNA. Mol. Cell. Biol. **7**:1296–1299.