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Genetics

An indicator gene to demonstrate intracellular transposition of defective retroviruses

(retrotransposition/mobile genetic element/Moloney murine leukemia virus/envelope gene)

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ABSTRACT **An indicator gene for detection and quantitation of RNA-mediated transposition was constructed (neo^{RT}). It was inserted into a Moloney murine leukemia provirus (Mo-MLV) deleted for the envelope gene to test for intracellular transposition of defective retroviruses [Mo-MLV(neo)^{RT}]. Neo^{RT} contains the selectable *neo* gene (which confers resistance to the drug G418), inactivated by a polyadenylylation sequence inserted between the *neo* promotor and coding sequence. The polyadenylylation sequence is flanked (on the antisense strand of the DNA) by a donor and an acceptor splice site so as to be removed upon passage of the provirus through an RNA intermediate. 3T3 cells transfected with the defective Mo-MLV(neo)^{RT} provirus are sensitive to G418. After trans-complementation with Mo-MLV, viral transcripts confer resistance to G418 upon infection of test cells. In the resistant cells, the polyadenylylation sequence has been removed, as a result in most cases of precise splicing of the intronic domain. Retrotransposition of the defective Mo-MLV(neo)^{RT} provirus was demonstrated by submitting transfected G418-sensitive clones to selection. Between 1 and 10 G418-resistant clones were obtained per 10^7 cells. Several possess additional copies, with evidence for precise removal of the intronic domain. By using target test cells in coculture experiments, extracellular intermediates of retrotransposition could not be detected.**

In eukaryotic organisms, several transposable elements are structurally highly homologous to proviruses (for review, see ref. 1). Elements such as *copia* in *Drosophila*, *Tyl* in yeast, or IAP sequences in mouse are 5- to 10-kilobase (kb) structures flanked by direct terminal repeats [300–600 base pairs (bp)] with a U3-R-U5 organization reminiscent of the long terminal repeat of retroviruses. They possess internal open reading frames related to *gag-pol*, with homologies to retroviral RNA-dependent DNA polymerase (2–5). In the case of the *Tyl* element, evidence has been provided that transposition requires passage through an RNA intermediate and reverse transcription (6). The homology between proviruses and "retrotransposons" (1, 6) is further strengthened by the occurrence of intracellular "virus-like particles" and of intermediates of reverse transcription in the case of *copia* and *Tyl* (7–11).

In mammals, analysis of transposition is difficult to perform by using the biological assays that have been developed in *Drosophila* or yeast (6, 12). Accordingly, putative transposons have been suspected from their structural similarities with proviruses and their high copy number in the genome. In the case of the IAP sequences, transposition was demonstrated by the discovery of their integration into unusual loci (13–15), a situation reminiscent of the retrovirus insertions observed in a number of tumors (16).

These observations raise a series of questions. First, how can it be demonstrated that putative transposons actually transpose in mammalian cells; in particular, are proviral copies of the retrovirus able to transpose? Second, is transposition modulated by genetic or epigenetic factors as observed in *Drosophila* and yeast (6, 17–19)? Third, can transposition be implicated in tumorigenic processes?

In addressing these questions, the potentially low frequency of transposition has to be taken into account (see refs. 20 and 21 for yeast and *Drosophila*). To analyze transposition in mammalian cells, we therefore constructed an "indicator gene for retrotransposition," which should allow the detection of transposition (by selective procedures) for any genetic element that transposes via an RNA intermediate.

MATERIALS AND METHODS

Plasmid Construction. To construct the retrotransposition indicator (neo)^{RT}, the sequence for polyadenylylation of the thymidine kinase (*tk*) gene of the herpes simplex virus was isolated from plasmid pAG0 (22) as a *Sma* I/*Nco* I 310-base-pair (bp) fragment and was inserted at the unique *Bam*HI site of pZip plasmid (23) between the Moloney murine leukemia virus (Mo-MLV) donor and acceptor splice sites after Klenow enzyme treatment of both fragments. The fragment containing the polyadenylylation sequence and the splice sites was next isolated as a *Kpn* I/*Kpn* I fragment and inserted between the SVtk promoter and the coding region of the *neo* gene at the *Bgl* II site of pSVtkneoβ (24) after Klenow treatment of both fragments; the neo^{RT} indicator gene (Fig. 1*A*) was then isolated as a *Hin*dIII/*Sma* I fragment.

To delete the *env* genes in pMov3 (25), the pMov3 *Eco*RI fragment was first cloned in the 3.4-kilobase *Eco*RI fragment of pSVtkneoβ; the *Bam*HI (bp 6537 in ref. 26)/*Cla* I (bp 7674 in ref. 26) fragment encoding part of the gp70 and the p15E proteins was then eliminated upon complete *Cla* I and partial *Bam*HI digestions.

The final construction [pMo-MLV(neo)^{RT}] was obtained upon ligation of the deleted pMov3 vector with the (neo)^{RT} indicator gene after Klenow treatment (Fig. 1*B*).

Cell Culture, Transfection, and Infection. Methods are described in ref. 27. Cells used were NIH 3T3 and FG10 (28), and the viral producer lines 3T3 Mo-MLV for Moloney and E40ψB for Mo-MLVneo (29). 3T3 LacZ cells were a gift from D. Rocancourt and C. Bonnerot (Institut Pasteur). Selection of G418-resistant cells was performed in the presence of geneticin (600 μg/ml) (G418), and cells containing

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Abbreviations: Mo-MLV, Moloney murine leukemia virus; neo, neomycin; env, envelope; tk, thymidine kinase.

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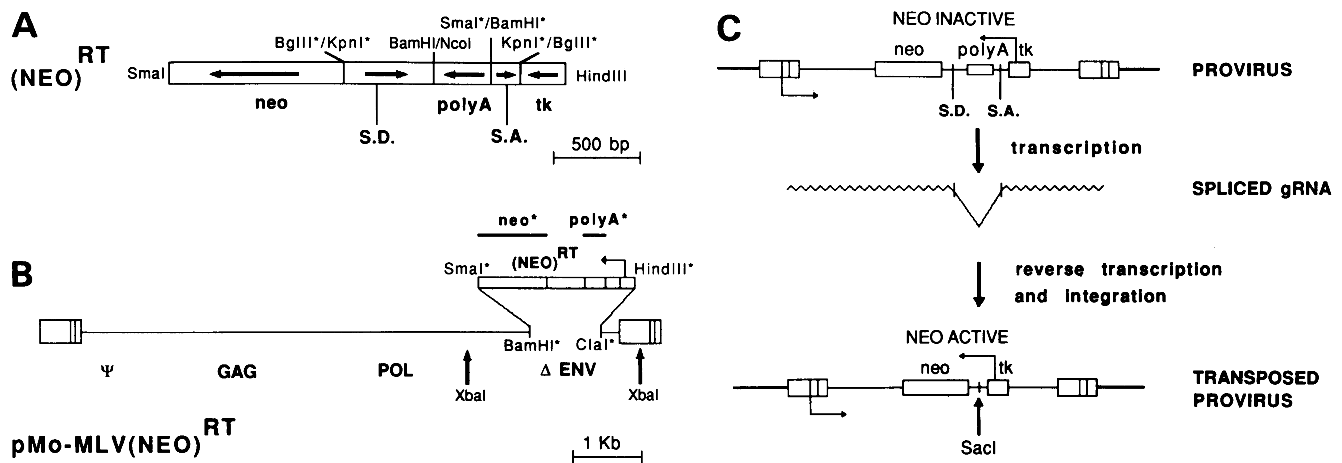

FIG. 1. Rationale of the test for detection of retrotransposition. (*A*) Structure of $(neo)^{RT}$, an indicator gene for retrotransposition. The orientation for each genetic element is indicated by arrows in boxes. tk, Promoter of the thymidine kinase gene of the herpes simplex virus; polyA, polyadenylylation sequence from the same gene; neo, coding sequence of the neomycin gene; S.D., splice donor; S.A., splice acceptor from Mo-MLV. (*B*) Structure of pMo-MLV$(neo)^{RT}$; the retroviral part of the construct is derived from the cloned pMov3 provirus; the neo and poly(A) fragments used as probes are indicated by bars. In *A* and *B*, the restriction sites used for the construction of the plasmids are marked with a star when lost. (*C*) Steps in the generation of an active *neo* structure from pMo-MLV$(neo)^{RT}$.

pSV2gpt (30) were selected in the presence of mycophenolic acid (25 μg/ml) and xanthine (250 μg/ml).

Cloning and Sequencing of the Splice-Site Junction. DNA from clone 3T3 gptMo$(neo)^{RT}$-A-5 (50 μg) was digested with *Xba* I and electrophoresed. DNA fragments (2.5–2.9 kb) were used to construct a partial genomic library in the λgt10 vector (31). Clones were screened by plaque filter hybridization with a ^{32}P-labeled *neo* probe. *Pvu* II/*Eco*RI fragments of positive inserts were subcloned in an M13mp10 vector and sequenced by the dideoxy chain-termination method (32).

RESULTS

Rationale of the Test. The principle of the test relies on the use of a selectable indicator gene that is activated by an RNA-mediated transposition when inserted in a retrotransposon (Fig. 1). In the indicator gene that we have developed [$(neo)^{RT}$; Fig. 1*A*], the selectable *neo* gene (tkneo) is inactivated by inserting between the promoter (*tk*) and the structural part of the gene (*neo*) a signal and site for polyadenylylation [poly(A)]; the sequence for polyadenylylation is flanked on its anti-sense DNA strand by a donor and an acceptor splice site. If $(neo)^{RT}$ is inserted in a retrotransposon in reverse orientation (Fig. 1 *B* and *C*), the intronic domain should be removed upon transcription of the retrotransposon and splicing of the RNA. The reverse transcription of this RNA should generate a DNA structure in which the sequence for polyadenylylation is removed, and a cell containing a retrotransposed copy should thus become resistant to G418 (Fig. 1*C*).

To test for nonviral retrotransposition of retroviruses, the $(neo)^{RT}$ gene was inserted in a cloned Mo-MLV provirus deleted for the genes encoding the major components of the viral envelope. Other regions, including the long terminal repeats, the packaging sequence, and the *gag-pol* genes were left unmodified to keep the possibility of nucleocapsid assembly, which may be required for reverse transcription of the RNA [pMo-MLV$(neo)^{RT}$; Fig. 1*B*].

The required characteristics for $(neo)^{RT}$ to be a retrotransposition indicator are thus (*i*) that expression of the selectable gene remains below the level required for resistance to G418 as long as the sequence for polyadenylylation in the intronic domain is present and (*ii*) that its removal creates a structure that confers resistance of the host cells to G418.

pMo-MLV$(neo)^{RT}$ Does Not Confer Resistance to G418. To prove that $(neo)^{RT}$ fulfills these requirements, it was introduced into NIH 3T3 cells by transfection of pMo-MLV-$(neo)^{RT}$. pSV2gpt was cotransfected to isolate clones in which pMo-MLV$(neo)^{RT}$ is nonselectively introduced [3T3 gptMo$(neo)^{RT}$ clones] and to measure transfection efficiencies. In a first test for the inactivation of the *neo* gene in neo^{RT}, the transfected cell population was directly submitted to G418 selection, and the number of resistant clones was compared to that obtained upon transfection of a plasmid in which the *neo* gene is active (pMo-MLVneo; ref. 29). pMo-MLV$(neo)^{RT}$ yielded only 0–3 G418-resistant clones per transfection (three experiments), whereas >83 G418-resistant clones were obtained with the pMo-MLVneo plasmid (a parallel selection for *gpt* expression in a medium containing mycophenolic acid indicated that transfection efficiencies were identical; Table 1). Analysis by Southern blot of the structure of five individual clones resistant to G418 (data not shown) revealed rearrangements (probably due to transfection) of the 3.5-kb *Xba* I fragment, which contains the indicator gene (indicated in Fig. 1*B*). In a second test, clones were selected for *gpt* expression, and seven of them that contained an unrearranged $(neo)^{RT}$ gene [see, for example, clones 3T3gptMo$(neo)^{RT}$-A and -B; see Fig. 3] were subjected to G418 treatment: none of them was G418 resistant (see also below). These experiments indicate that the $(neo)^{RT}$ gene does not confer resistance to G418 unless rearranged.

Viral Transcripts of pMo-MLV$(neo)^{RT}$ Confer Resistance to G418. We expected a fraction of the RNA produced by pMo-MLV$(neo)^{RT}$ in the transfected cells to be spliced and therefore to lack the polyadenylylation sequence. This RNA should retain the features of a viral genomic RNA, which upon trans-complementation for the viral products should be packaged into viral particles. To test whether reverse-transcribed copies (Fig. 1*C*) can confer resistance to G418, the supernatant of 3T3 gptMo$(neo)^{RT}$ cells infected with wild-type Mo-MLV virus was transferred to NIH 3T3 test cells. For the seven transfected 3T3 gptMo$(neo)^{RT}$ clones mentioned in the previous section, colonies resistant to G418 were obtained. The titer of the supernatants varied from 0.5 to 2 × 10^3 neo^{R}fu per ml. The structure of the new proviruses was analyzed by Southern blot.

As shown in Fig. 2*A*, the $(neo)^{RT}$-containing *Xba* I fragment observed in the 3T3 gptMo$(neo)^{RT}$ clones with the *neo* probe has a reduced size in the infected G418-resistant cell

Table 1. Test for *neo* inactivation in pMo-MLV $(neo)^{RT}$

DNA transfected	Number of clones resistant to G418	Number of clones resistant to mycophenolic acid
pMo-MLV$(neo)^{RT}$ + pSV2gpt	3, 0, 2	95, 72, 86
pMo-MLVneo + pSV2gpt	125, 83, 97	87, 65, ND

DNA (10 μg) containing equimolar amounts of pSV2gpt and pMo-MLV$(neo)^{RT}$ or pMo-MLVneo (29) was transfected into 10^5 NIH 3T3 cells by the calcium phosphate precipitation technique; a fraction of the cells was subjected to selection in either G418 or mycophenolic acid plus xanthine. Values are shown for each of three independent experiments; ND, not determined.

population [Mo$(neo)^{RT}$ pop] or in individual subclones [Mo$(neo)^{RT}$ cl 1 to cl 7]. In the population, up to four bands of unequal intensity were observed, two of them (2.7 and 2.6 kb long) being most frequently represented in subclones. Among 25 G418-resistant infected clones analyzed, 8 contained the 2.7-kb band and 12 contained the 2.6-kb band. The other two bands observed were smaller (2.5 and 2.0 kb); the original 3.5-kb band was never observed. None of the four bands hybridized with the poly(A) probe of the intronic domain (Fig. 2*B*).

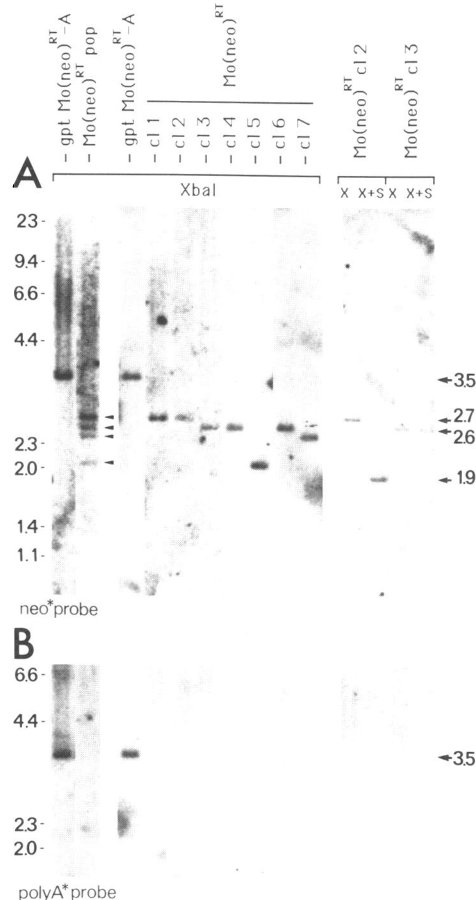

FIG. 2. Southern blot of genomic DNA from G418-resistant cells infected with virus rescued from pMo-MLV$(neo)^{RT}$-transfected cells. Cells from 3T3 gptMo$(neo)^{RT}$-A [a 3T3 clone obtained by cotransfection of pSV2gpt and pMo-MLV$(neo)^{RT}$ that contains only one proviral copy] were infected with Mo-MLV viruses; after 7 days the supernatant was collected, and 100 μl was used to infect 10^4 3T3 test cells; 2 days later cells were submitted to G418 selection. G418-resistant cells were analyzed as a mixed clone population [3T3 Mo$(neo)^{RT}$ pop] or as individual clones [3T3 Mo$(neo)^{RT}$ cl 1–7]. Restriction enzymes used were *Xba* I (X), or *Xba* I and *Sac* I (X+S) to test for correct splicing. Ten or 25 μg (two first lanes) of DNA was used. Filters were hybridized with either the *neo* probe (*Bgl* II/*Sma* I fragment; *A*) or the poly(A) probe (*Sma* I/*Nco* I fragment; *B*).

To characterize further the 2.7-kb band, whose size corresponds to that expected for precise splicing, we tested whether it contains the *Sac* I site, which should be generated at the splice site junction (ref. 33; Fig. 1*C*). The 2.7-kb fragment was indeed reduced in size to 1.9 kb when the genomic DNA was digested by *Xba* I and *Sac* I restriction enzymes and probed using the *neo* fragment. In contrast, the 2.6-kb band does not contain a *Sac* I site, and it may correspond to splicing events involving cryptic D-A sites or to rearrangements during reverse transcription. Thus, passage of the original pMo-MLV$(neo)^{RT}$ through an RNA intermediate generates structures in which the *neo* gene is active.

The Defective Mo-MLV$(neo)^{RT}$ Provirus Retrotransposes. Cells (10^8) from four independent 3T3 gptMo$(neo)^{RT}$ clones were subjected to G418 selection. In each case, 1–10 G418-resistant clones per 10^7 cells were obtained, and these were expanded for DNA analysis (examples shown in Fig. 3). They can be grouped into two categories.

In the first group (13 clones of 37 G418-resistant clones analyzed), the 3.5-kb *Xba* I fragment (corresponding to the transfected plasmid; Fig. 1*B*) was replaced by a fragment (Fig. 3*A*, cl A-1 and cl A-2) that did not hybridize with the poly(A) intronic probe (Fig. 3*B*); these clones are interpreted as rearrangements of the initial copies involving a deletion of at least the poly(A) sequence.

In the second group (24 of 37 clones), one and sometimes two bands hybridizing to the *neo* probe were observed together with the initial 3.5-kb band of the transfected clones (Fig. 3*A*, clones A-3 to A-5 and B-1). These additional bands do not hybridize with the poly(A) probe (Fig. 3*B*). In several cases (respectively, 5 and 2 clones), they comigrate with either the 2.6- or the 2.7-kb spliced copies of the infected clones (Fig. 3*A*, clones A-5 and B-1).

The 2.7-kb band was identified as originating from reverse transcription of a correctly spliced transcript of the transfected provirus by DNA digestion using *Xba* I and *Sac* I, which generates a 1.9-kb band (Fig. 3*A*, clone A-5). This conclusion was confirmed by cloning the 2.7-kb *Xba* I fragment from one of the G418-resistant clones (clone A-5) and sequencing the splice site junction (Fig. 3*C*).

There Are No Extracellular Intermediates for Defective Mo-MLV$(neo)^{RT}$ Provirus Transposition. Two series of experiments have been performed to test for extracellular intermediates (such as nucleocapsids or viral particles) in the generation of the retrotransposed Mo-MLV$(neo)^{RT}$ copies.

First, supernatants from four independent transfected clones and from four clones containing a retrotransposed copy derived from them were tested for the presence of *neo* colony-forming viral particles by infection of 3T3 cells followed by selection in G418. In no instances could any resistant clone be detected (100 ml of supernatant tested per clone). We also verified that the supernatants did not contain wild-type Mo-MLV, by using the F^+L^- test, which detects N, B, and NB viruses (28).

Second, 3T3 gptMo$(neo)^{RT}$ clones were expanded in the presence of marked test cells that could be a target for extracellular intermediates. $LacZ^+$ NIH 3T3 cells, which can be identified by a β-galactosidase *in situ* enzyme assay (34), were used. After up to 3 weeks of coculture, G418 selection was applied. For the two transfected clones tested, 18 and 25 G418-resistant clones were obtained (Fig. 4), but none of them was $LacZ^+$. We verified that the $LacZ^+$ cells are infectable by recombinant Mo-MLV retroviruses and selectable in G418 by infecting cells of the coculture with Mo-MLVneo virus produced in ψ-2 (an N, B tropic helper

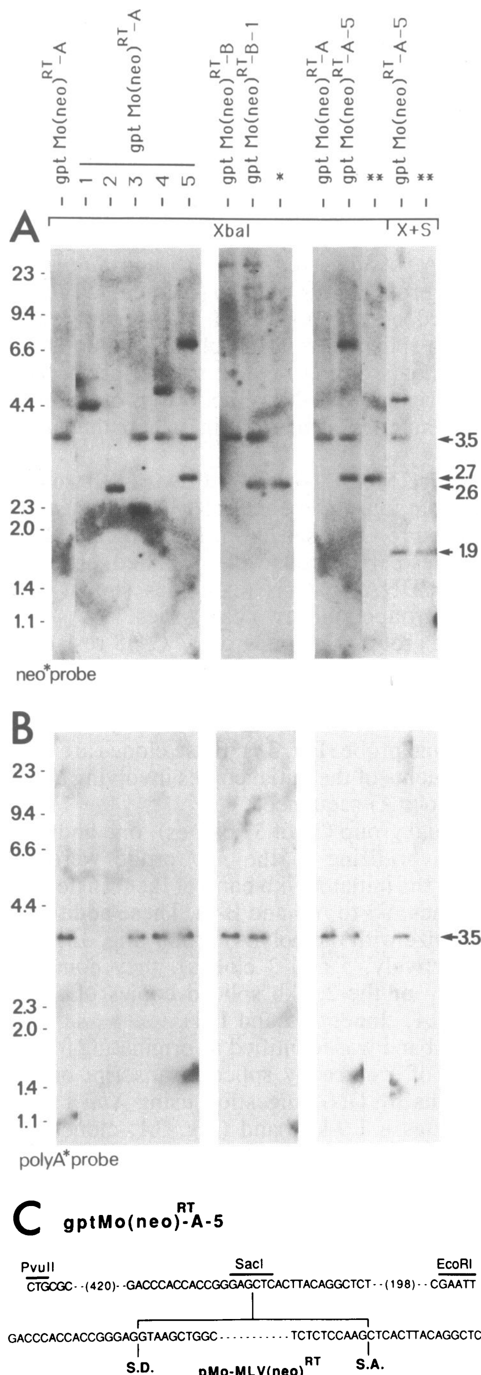

FIG. 3. Southern blot analysis of genomic DNA from G418-resistant cells isolated from 3T3 gptMo(neo)^{RT}-transfected clones; sequence of a splice junction. (*A* and *B*) Cells (10^7) from clones 3T3 gptMo(neo)^{RT}-A and -B were subjected to G418 selection at 10^6 cells per 10-cm plate. Clones resistant to G418 (0–2 per plate) were obtained [see, for example, 3T3 gptMo(neo)^{RT} cl A-1 to A-5 and cl B-1]; DNA (10 μg) was analyzed as described in Fig. 2 after restriction with *Xba* I and *Xba* I plus *Sac* I to test for correct splicing. Comigration of the new copies in cl B-1 and cl A-5 with, respectively, the 2.6- and the 2.7-kb *Xba* I fragments of clones Mo(neo)^{RT} cl 3 (*) and cl 2 (**) of Fig. 2. Filters were hybridized with the neo probe (*A*) or the poly(A) probe (*B*). (*C*) Splice junction in a transposed proviral copy of pMo-MLV(neo)^{RT}. The 2.7-kb *Xba* I fragment from 3T3 gptMo(neo)^{RT}-A-5 DNA was cloned in λgt10, and a 662-bp fragment from a *Pvu* II site in the *neo* coding region to the *Eco*RI site in the *tk* promoter was subcloned in M13 and sequenced. The newly generated *Sac* I site at the splice junction is indicated. The published sequences of the donor and acceptor regions in pMo-MLV(neo)^{RT} (lower part) are from ref. 26.

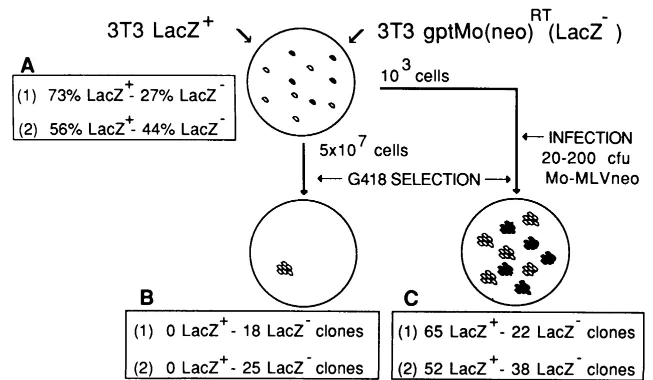

FIG. 4. Test for extracellular intermediates of retrotransposition. Cells from clones 3T3 gptMo(neo)^{RT}-A (1) and -B (2) were mixed with 3T3 LacZ$^+$ cells and expanded for 10–21 days. Then a fraction of the cells was stained for β-galactosidase and counted (*A*), 5×10^7 cells were plated at 2×10^6 cells per 10-cm plate (*B*), and 10^3 cells were infected with 20–200 colony-forming units of Mo-MLVneo to test for infectivity and selection in G418 of both cell lines (*C*). G418 was applied for 15 days (*B* and *C*), and the resistant clones were stained.

cell line, ref. 35) at a low multiplicity before G418 selection. LacZ$^+$ clones resistant to G418 were obtained in a proportion almost identical to that of LacZ$^+$ cells in the coculture (Fig. 4). The absence of LacZ$^+$ clones resistant to G418 upon direct selection thus suggests that there are no extracellular intermediates involved in the detected retrotransposition.

DISCUSSION

The *neo*^{RT} Gene as an Indicator for Retrotransposition. A method to detect and quantitate RNA-mediated transposition in eukaryotic cells is described. It relies on use of an engineered gene (*neo*^{RT}), whose expression should be selectable upon reverse transcription of an RNA from its noncoding strand. In the (*neo*)^{RT} construct, the unit of transcription of the *neo* gene is interrupted by a sequence containing a signal of polyadenylylation, and this sequence is flanked on the antisense strand of the DNA by a donor and an acceptor splice site. If the *neo*^{RT} gene is introduced into a retrotransposon in such an orientation that the splicing sites are on the DNA strand transcribed from the promoter of the transposon, a retrotransposed DNA copy is expected to produce (from the antisense strand) a translatable neo mRNA (Fig. 1).

The *neo*^{RT} construct does not confer resistance to G418 unless rearranged. First, upon transfection of pMo-MLV-(neo)^{RT} into 3T3 cells, only a few cells are directly selectable in G418 medium as compared to the number obtained with pMo-MLVneo DNA (Table 1); furthermore, these cells always disclose rearrangements of the *neo*^{RT} structure (we do not know whether they correspond to events of transposition or to mutations associated with the transfection). Second, cells containing unrearranged *neo*^{RT}, among the gptMo-MLV(neo)^{RT} clones obtained by cotransfection, are all sensitive to G418: after clonal expansion and selection in G418, only 1–10 cells per 10^7 are G418 resistant; again, the G418-resistant cells always disclose a rearranged *neo*^{RT} gene, in which the polyadenylylation region has been removed (Fig. 3). Third, G418-resistant cells obtained upon infection with the rescued provirus always harbor a deletion of the polyadenylylation sequence (Fig. 2).

We also present evidence that reverse-transcribed copies of the antisense strand of *neo*^{RT} confer resistance to G418. These were obtained upon infection of pMo-MLV(neo)^{RT}-transfected cells with Mo-MLV virus for trans-complemen-

tation: recombinant retroviruses were produced that confer resistance to G418 upon retroviral infection of 3T3 test cells.

In preliminary experiments, we constructed four different retrotransposition indicator genes, using the thymidine kinase or the β-globin polyadenylylation sequences, and two donor–acceptor splice site-containing sequences, derived from the Mo-MLV or from the simian virus 40 late region. Although both polyadenylylation sequences resulted in the effective inactivation of the *neo* gene when associated with Mo-MLV-derived splice sequences, they were much less effective when associated with the simian virus 40-derived splice sequences. Although several factors could be involved in polyadenylylation efficacy and/or RNA stability (36), it remains possible that the potent blockade obtained in the neo^{RT} gene finally selected combines transcriptional and translational inhibitions. A second unexpected result is that the viruses rescued from the 3T3 gptMo(neo)^{RT} cells gave several (at least three) proviral structures in addition to the expected one. Molecular cloning will indicate whether they derive from alternative splicing or from modifications during reverse transcription. A more complete understanding of the mechanisms involved could help in designing other indicator genes.

Thus, although the neo^{RT} gene may still not be the optimal structure for quantitative analysis, it has all the properties required for an indicator of retrotransposition.

Retrotransposition of a Defective Mammalian Provirus. neo^{RT} was introduced into a Mo-MLV provirus that is expected to be defective because of the deletion of its *env* gene. Indeed, in the supernatant of several 3T3 gptMo-(neo)^{RT} clones, we could not detect any extracellular intermediates capable of conferring resistance to G418 either by direct transfer of the supernatants to the same NIH 3T3 cell line or by cocultivating them with a LacZ$^+$ NIH 3T3 tester cell line. Clones resistant to G418 were nevertheless derived by selection from these cells. In each case, a modified neo^{RT} gene was found in addition to the original copy or alone. Among 37 independent G418-resistant clones analyzed, 2 were unambiguously identified as resulting from reverse transcription of a correctly spliced RNA. We suspect that many others may also correspond to retrotransposition events, as 24 of them have gained a new neo^{RT} copy. These copies might correspond to reverse transcription of RNA of unexpected structure. For instance, the structure generating a 2.6-kb *Xba* I fragment was also found in G418-resistant cells infected with a rescued provirus. DNA rearrangements may account for the other cases, in particular in cells in which the original copy is missing. Finally, it should be noted that in three cases we found two additional neo^{RT} copies in G418-resistant clones. Future evaluation of the significance of these unexpected structures will have to take into account that transposition (*i*) might be highly mutagenic for the retrotransposon as observed in yeast for *Ty1* (6) and (*ii*) under given circumstances might be a frequent and/or cooperative event as observed in *Drosophila* (17–19).

The results presented here suggest that a provirus deleted for the *env* gene can generate retrotransposed copies that integrate at new positions in the genome without extracellular intermediates. This conclusion reinforces the postulated similarities between proviruses and retrotransposons of lower eukaryotes, for which neither *env*-associated open reading frames nor extracellular intermediates of retrotransposition have been detected (reviewed in ref. 1). Furthermore, transposition of defective proviruses might play an important role in the fluidity of the genome of eukaryotic cells and in problems related to mutagenesis and oncogenicity.

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