JOURNAL OF VIROLOGY, Mar. 1992, p. 1571–1578
0022-538X/92/031571-08$02.00/0

Vol. 66, No. 3

High-Frequency Intracellular Transposition of a Defective Mammalian Provirus Detected by an In Situ Colorimetric Assay

THIERRY TCHENIO AND THIERRY HEIDMANN*

Unités de Biochimie-Enzymologie et de Physicochimie Macromoléculaire, Centre National de la Recherche Scientifique U147, and Institut National de la Santé et de la Recherche Medicale U140, Institut Gustave Roussy, 39 rue Camille Desmoulins, 94805 Villejuif Cedex, France

Received 25 July 1991/Accepted 4 December 1991

We devised an indicator gene for retrotransposition, nlsLacZ^{RT}, which contains the *Escherichia coli lacZ* gene fused to a nuclear location signal (nlsLacZ), engineered in such a way that the gene is expressed only if the structure in which it has been inserted transposes itself through an RNA intermediate. A cloned murine leukemia retrovirus with an ecotropic host range (Moloney murine leukemia virus), rendered defective by a large deletion encompassing the three viral *gag*, *pol*, and *env* open reading frames, was marked with this indicator gene and introduced by transfection into heterologous feline cells. No β-galactosidase activity could be detected among the clonal cell population, unless the defective provirus was complemented in *trans* by the *gag-pol* gene products. Under these conditions, cell variants which disclosed an easily detectable nuclear blue coloration upon in situ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining were observed. Fluorescence-activated cell sorting of the β-galactosidase-positive cells, followed by Southern blot analysis, demonstrated an unambiguous correlation between nlsLacZ^{RT} activation and retrotransposition of the marked provirus. Transposition occurs at a high frequency (up to 10^{-4} events per cell per generation), which is dependent on the level of expression of the *gag-pol* gene and is concomitant with the release of noninfectious retroviruslike particles which are the hallmarks, but not the intermediates, of the intracellular transposition process.

It is well documented that in mice many neoplasms originate from mutagenic insertions of mobile genetic elements in critical loci that control the proliferation state of the cell. Insertion may result either from intracellular transposition of endogenous retrotransposons (reviewed in references 6, 11, 15) or from infection of the cells by horizontally transmitted retroviruses (reviewed in references 24, 29). There is, possibly, no fundamental difference between these two classes of mobile genetic elements, which share large structural homologies and are both associated with production of virus (or viruslike) particles: for retrotransposons such as *Saccharomyces cerevisiae* Ty1 (reviewed in reference 3), *Drosophila* copia (reviewed in reference 7), or mouse IAP (reviewed in reference 16), these are strictly intracellular, whereas infectious viral particles are exported out of the cell and can be horizontally transmitted. Furthermore, it has been recently shown that a Moloney murine leukemia retrovirus (MoMLV) rendered defective for production of infectious viral particles by deletion of its envelope gene—and whose structure thus resembles that of classical retrotransposons—is also capable of intracellular transposition in murine or human cells (13, 28).

To detect and analyze these potentially mutagenic processes in a quantitative manner, we had previously developed an indicator gene for retrotransposition (13) which contains the neomycin (*neo*) selectable gene, which encodes resistance to G418 in mammalian cells. This gene is engineered in such a way that it can be expressed only if the mobile element in which it has been inserted transposes itself through an RNA intermediate, i.e., retrotransposes. Although this indicator gene has proven to be very effective in detecting retrotransposition events in cells in culture (even when the frequency was as low as 10^{-7} event per cell per generation) and to select those cells in which the events had occurred (12, 13), it might not be adapted to future in vivo studies that should be performed in transgenic mice; indeed, the presence of an activated *neo* gene cannot be easily detected unless the tissues are first established in culture, which should be extremely tedious and often not even possible.

We have therefore devised another indicator gene, whose rationale is close to that of the previously reported *neo*^{RT} gene (13), but which makes use of the *Escherichia coli* β-galactosidase (*lacZ*) gene fused to the nuclear location signal of the simian virus 40 large T antigen as a reporter (nlsLacZ; 4, 14). The nlsLacZ gene is particularly adapted to refined in situ analysis, since its activity, targeted to the nuclear membrane, can be unambiguously evidenced by cytochemical staining at the individual-cell level. Accordingly, here we describe and characterize an nlsLacZ-based indicator gene, nlsLacZ^{RT}, that we introduced into a cloned murine leukemia retrovirus with an ecotropic host range (MoMLV) rendered defective by deletion of the three canonical retroviral *gag*, *pol*, and *env* open reading frames (ORFs). After transfection into heterologous feline cells, no β-galactosidase expression was detected unless the *gag* and *pol* gene products, which are necessary for transposition of the provirus (28), were provided in *trans*. Under these conditions, β-galactosidase expression was easily detected either after 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining or after loading with a noncytotoxic, fluorescent β-D-galactosidase substrate, namely, fluorescein-β-D-galactopyranoside (FDG; 23), which allowed isolation of viable marked cells by fluorescence-activated cell sorting (FACS). In the latter case, FACS sorting of the β-gal$^+$ cells, followed by Southern blot analysis, unambiguously demonstrated that β-galactosidase expression is actually linked to retrotransposition of the marked provirus.

* Corresponding author.

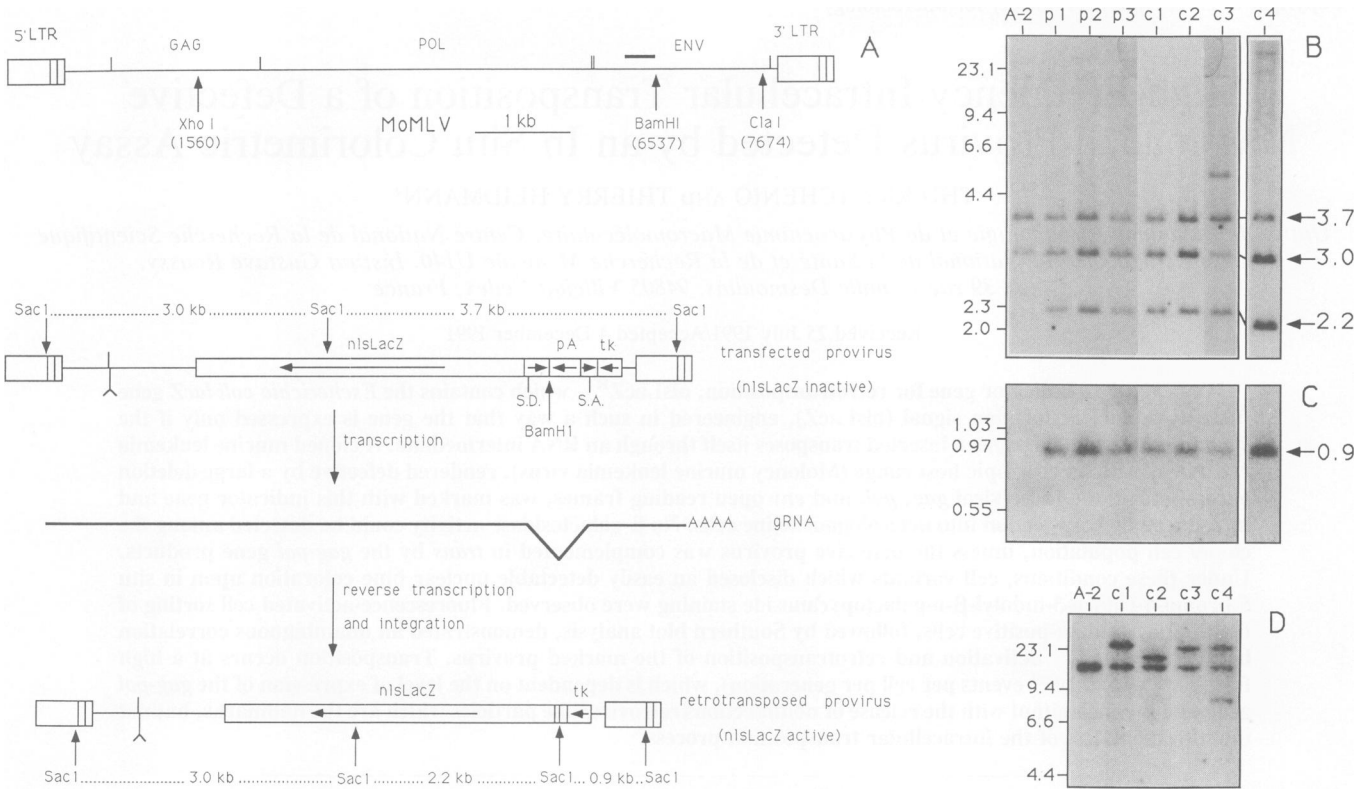

FIG. 1. Structure and retrotransposition of the marked nlsLacZ^{RT} provirus. (A) Structure of the marked provirus and rationale of the test. The indicator gene is represented by open boxes, with an arrow indicating the orientation of each genetic element. Abbreviations: tk, promoter of the herpes simplex virus thymidine kinase gene; nlsLacZ, ORF of the *lacZ* gene with the nuclear location signal (nls) from simian virus 40 large T; SD and SA, splice donor and acceptor sites, respectively, from MoMLV; pA, polyadenylation sequence from the herpes simplex virus thymidine kinase gene. The marked defective provirus was derived from a cloned MoMLV provirus (top line; pMov3 [10]) upon deletion from *Xho*I (1560) to *Cla*I (7674). The numbers refer to distances from the RNA cap site. The terminal LTRs and the AUG codon of the remaining part of the *gag* ORF, with the inactivating frameshift (indicated as ⅄, an 8-bp *Sac*II linker at position 623 [initially constructed by S. Goff]), are indicated. The provirus for *gag-pol* gene transcomplementation was derived from the cloned MoMLV upon *env* gene deletion from *Bam*HI (6537) to *Cla*I (7674). The steps for generation of an active nlsLacZ gene by retrotransposition of the transfected marked provirus are indicated schematically, along with the splicing of the proviral RNA intermediate and the resulting removal of the inactivating pA sequence in the transposed copy. Restriction sites for *Sac*I and the lengths of the corresponding fragments in both the initial and transposed proviruses are indicated. (B to D) Southern blot characterization of the retrotransposition of the nlsLacZ^{RT} marked provirus. Genomic DNAs of the transcomplemented A-2 clone (lane A-2), three different FDG^{+} cell populations sorted by FACS (lanes p1, p2, and p3), and clones derived by ultimate dilution from p1 (lanes c1 to c4) were restricted with either *Sac*I (B and C) or *Bam*HI (D) and hybridized with ^{32}P-labelled nlsLacZ (B and D) or tk gene (C) fragments. The bar above the *env* segment of the top line indicates the probe used to detect *gag-pol* transcripts in Fig. 4.

Transposition of the marked provirus occurred at a frequency at least 100 times higher than previously reported (13, 28) and was concomitant with release by the cells of noninfectious retroviral particles, whose number can be as large as those encountered in productive infections by wild-type MoMLV. These extracellular particles are the hallmarks, but not the intermediates, of the intracellular retrotransposition process.

MATERIALS AND METHODS

DNA constructions. All plasmids were constructed by standard cloning procedures; numbers refer to distances from the viral MoMLV cap site.

(i) Construction of the defective nlsLacZ^{RT} marked provirus. The defective nlsLacZ^{RT} marked provirus plasmid (Fig. 1A) was derived from the previously described MoMLV neo^{RT} provirus (13), which was first recloned into the MoMLVneo plasmid (25) by homologous substitution of the proviral domains (after enzyme restriction at unique sites in the long terminal repeats (LTRs) (12a). A frameshift at the beginning of the *gag* ORF (an 8-bp *Sac*I linker at position 623 [initially constructed by S. Goff]) was introduced by a three-fragment ligation, including two fragments derived from the modified MoMLVneo^{RT} plasmid described above, namely an *Xho*I (unique site in the provirus at position 1560)-*Cla*I (unique site in the pBR322 sequence) fragment and a *Cla*I (unique site in the pBR322 sequence)-*Xba*I (position −151 in the 5′ LTR) fragment and an *Xba*I (position −151 in the 5′ LTR)-*Xho*I (unique site in the provirus at position 1560) fragment (from plasmid pA5, a gift from J. Murphy) homologous to that described above but containing the indicated frameshift in the *gag* gene, thus yielding MoMLVneo^{RT}-1. For reduction of the splice donor-containing fragment of the indicator gene, it was excised as a 260-bp *Mbo*I (161)-*Mbo*I (420) fragment from the previously described *neo*^{RT} gene (13) and subcloned twice in pSP64 (Promega), first at the unique *Bam*HI site in the polylinker,

and then as a *Sac*I Klenow-treated *Sal*I fragment (both restriction sites in the polylinker) inserted in the pSP64 vector opened by *Bam*HI-Klenow treatment and *Sal*I. The splice donor-containing sequence was then excised as a *Sal*I-*Bam*HI fragment, and a second proviral intermediate, MoMLVneo^{RT}-2, was constructed upon three-fragment ligation with a *Cla*I (unique site; see above)-*Sal*I (position 3705 in the provirus) fragment and a *Bam*HI (at the junction between pA and D in the indicator gene)-*Cla*I (same as above) fragment, both from MoMLVneo^{RT}-1. The nlsLacZ gene was excised from pGEMnlsLacZ (4) as a *Bam*HI-Klenow-treated *Sal*I fragment and inserted into MoMLVneo^{RT}-2 restricted by *Xho*I-Klenow treatment and *Sal*I, thus yielding the final construct in Fig. 1.

(ii) Construction of the plasmid for *gag-pol* transcomplementation. The plasmid for *gag-pol* transcomplementation was derived from a subcloned pMov3 provirus (described in reference 13), in which the pBR322 *Cla*I site had been removed, after complete *Cla*I and partial *Bam*HI digestions, which resulted in deletion of the envelope gene from positions 6535 to 7674.

Cell and nucleic acid analysis. G355-5 feline cells (a gift of J. F. Nicolas) were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (GIBCO) at 37°C in 5% CO_2. Transfections of G355-5 cells were achieved by the calcium phosphate method. Cells cotransfected with pSV2gpt (20), pBSpac (5), or pSVtkneoβ (22) were selected in the presence of mycophenolic acid (25 μg/ml) plus xanthine (250 μg/ml), puromycin (2 μg/ml), and G418 (700 μg/ml), respectively. Cellular RNA and DNA extractions and Northern and Southern blot analyses were performed as described in reference 12. When indicated, the autoradiograms were scanned with a Chromoscan densitometer.

X-Gal staining, FDG loading, and FACS. X-Gal staining was performed as described in reference 26. The reaction was allowed to proceed at 42°C for 7 h; cells with blue nuclear coloration were scored by microscopic examination. The protocol for FDG loading was similar to that described in reference 23, except for the two following modifications, made to reduce the high background level of endogenous lysosomal β-D-galactosidase activity in the G355.5 cells. (i) Cells were incubated for 5 min in 2% dimethyl sulfoxide at 37°C before FDG loading, and (ii) 2 min after FDG loading, enzymatic conversion of FDG to fluorescein was stopped upon addition of a competitive inhibitor, phenylethylthiogalactoside, at a final concentration of 1 mM. Cells were sorted with a FACS 440 (Becton Dickinson) or an EPICS (Coulter) cell sorter; they were immediately plated in 24-well culture plates (5×10^3 to 10^4 FDG$^+$ cells), maintained for 15 h in RPMI 1640 medium with 10% fetal calf serum–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.3)–10 μg of gentamicin per ml; and then expanded in normal growth medium.

Infectivity test and RTase assay. Supernatants were harvested from cell cultures close to confluency (5×10^6 to 10^7 cells in 10-cm-diameter culture dishes with 10 ml of growth medium), filtered through 0.22-μm-pore-size filters, and immediately tested or stored at −70°C until use. To search for infectious extracellular intermediates, 10 ml of supernatant (plus, when mentioned, Polybrene at a final concentration of 5 μg/ml) was left in contact with feline G355-5 test cells (5×10^5 cells plated in a 10-cm-diameter culture dish on the day before) for 24 h; cells were maintained in culture for another day in fresh medium and then fixed and stained by the X-Gal procedure. Reverse transcriptase (RTase) activity was determined from 10 μl of supernatant in a standard assay mixture as described in reference 8 (except for the Mn^{2+} concentration [0.6 mM instead of 0.2 mM]). [^{3}H]TTP incorporation was measured after trichloroacetic acid precipitation of the DNA products on glass fiber filters (GF/C; Whatman) and was linear over at least a 60-min period. RTase activity was derived from the slope of the line.

RESULTS

Construction of cell lines containing an nlsLacZ^{RT} marked provirus. nlsLacZ^{RT} was derived from the previously described *neo*^{RT} indicator gene (13) by replacing the *neo* coding sequence by that of the nlsLacZ gene (4) and by further reducing the length of the fragment containing the splice donor of the indicator gene (see Materials and Methods). This structure was inserted into a cloned murine leukemia retrovirus with an ecotropic host range (MoMLV) that was rendered defective by deleting 6.1 kb of the internal sequence encompassing the three canonical *gag*, *pol*, and *env* viral ORFs and by introducing a frameshift 3′ to the initiation codon of the remaining *gag* fragment (Fig. 1A). This marked proviral structure, despite the size of the nlsLacZ^{RT} indicator gene (4.5 kb), should still generate a genomic RNA whose length (6.7 kb) and structure (it still contains the *cis*-required extended Ψ sequence [1, 2]) are compatible with encapsidation into a core particle.

This marked defective provirus was introduced into G355-5 feline cells by cotransfection with pSV2gpt (20), and mycophenolic acid-resistant clones were selected. Feline cells were chosen because (i) the probability that an infectious retrovirus would be produced by homologous recombination or by complementation with endogenous retroviruses is lower in heterologous cells than in murine cells and (ii) MoMLV provirus expression in this cell line is nearly as efficient as in murine NIH 3T3 cells (data not shown). Two clones (A and B) were isolated that contained one full-length copy of the transfected proviral structure and unaltered nlsLacZ^{RT} structures, as evidenced by Southern blot analysis (see below). X-Gal staining of more than 10^7 cells from each clone failed to reveal any β-gal$^+$ cells (Fig. 2A), which proves that the transfected nlsLacZ^{RT} gene does not spontaneously confer the β-gal$^+$ phenotype, as expected.

Transcomplementation with the *gag-pol* gene generates β-gal$^+$ cells. The fact that A and B cells do not spontaneously segregate β-gal$^+$ cell variants is consistent with our preliminary observations that MoMLV's *gag-pol* gene products are required for intracellular retrotransposition of the marked provirus (28). A plasmid containing the MoMLV *gag-pol* gene under the genuine LTR-contained promoter and polyadenylation signal was therefore introduced by cotransfection with either pBSpac (5) or pSVtkneoβ (22), which confer resistance to puromycin and G418, respectively. Several clones were derived from clone A (seven G418^r and eight Pur^r clones), which were tested for segregation of β-gal$^+$ variants. X-Gal staining about 1.5 months after transfection (~45 cell generations) disclosed β-gal$^+$ cell variants for three G418^r and seven Pur^r clones (Fig. 2B shows one of the transcomplemented clones), with an apparent frequency ranging from 10^{-6} to more than 10^{-3} of the cells (only one G418^r clone against six Pur^r clones segregated β-gal$^+$ variants at a high frequency [$>10^{-3}$], possibly because of selection for higher levels of expression of the transfected *gag-pol* gene by pBSpac and puromycin selection [see below]). Southern blot analysis of the genomic DNA from all positive clones demonstrated stable integration of one to five copies of the transfected *gag-pol* gene

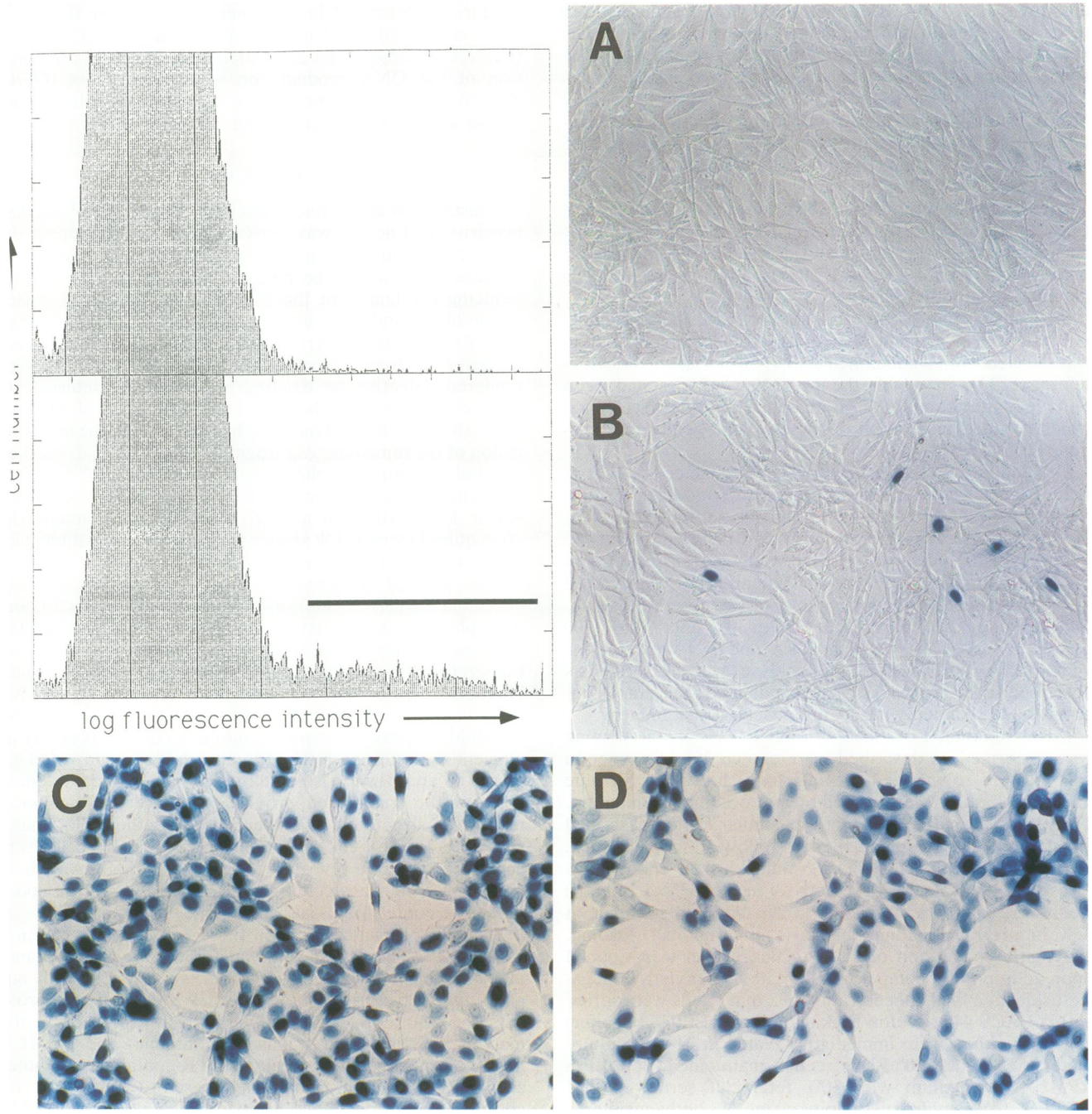

FIG. 2. Evidence for β-galactosidase-positive cell variants in *gag-pol*-transcomplemented clones and FACS. Inset, cytofluorometric analysis of transcomplemented A-2 cells (lower part) and control A cells (upper part) after FDG loading as described in Materials and Methods. The cell fractions marked with a bar were sorted and collected as populations (FDG^+ cells), and in these experiments they represent 0.6% ± 0.2% of the total number of A-2 cells counted (1×10^6 to 2×10^6 cells). (A to D) Histochemical X-Gal staining of control A cells (A), A-2 cells (B), FDG^+ cells sorted by FACS (p1; C), and a clone (c1; D) derived by limiting dilution from p1.

(data not shown). One of the transcomplemented clones producing $\beta\text{-gal}^+$ variants at a high frequency (A-2) was selected for further detailed studies.

To characterize the $\beta\text{-gal}^+$ cells, they were sorted by FACS (Fig. 2). The sorting procedure relies upon the use of FDG, which is cleaved by β-galactosidase to yield fluorescein, which can be optically detected. As illustrated by the FACS profile in Fig. 2 for the cells from the A-2 clone (inset, bottom), two cell populations were distinguished: more than 99% of the cells segregated in a peak of low fluorescence intensity, which further corresponds to the profile obtained with the control cells without *gag-pol* transcomplementation (clone A cells; Fig. 2, inset, top), and a small peak of higher fluorescence intensity observed only with the transcomplemented clones, which represented 0.6% ± 0.2% of the cells in the experiment shown in Fig. 2 (three independent cell

sortings). These cells were collected as a mixed population (FDG^+ cells) from which subclones were also derived by limiting dilution. X-Gal staining, as illustrated in Fig. 2C, disclosed that actually more than 90% of the FDG^+ cells were X-Gal^+, with a large fluctuation in staining intensity, from nearly colorless to dark blue. This heterogeneity cannot be attributed to clonal heterogeneity, since it was also observed within subclones (Fig. 2D). The percentages of β-gal^+ cells as scored by FACS in three independent experiments were close to the values obtained by X-Gal staining (FACS, 0.6% ± 0.2%; X-Gal staining, 0.7% ± 0.1%). Although they were less extensively studied, similar results were obtained with clones A-3 and A-4.

β-Gal^+ cells contain retrotransposed copies of the marked provirus. The DNA of the FDG^+ cells sorted by FACS was analyzed on Southern blots to determine whether the FDG^+ phenotype is actually associated with retrotransposition of the marked provirus. As schematized in Fig. 1, genomic DNA restriction with *Sac*I should reveal two fragments for the transfected copies upon hybridization with an nlsLacZ probe: a 3.7-kb fragment and a 3.0-kb fragment, encompassing the whole proviral structure; these were indeed observed (Fig. 1B, lane A-2). Upon retrotransposition with precise splicing at the donor and acceptor splice sites of the indicator, a new restriction site for *Sac*I should be generated at the splice junction, as previously noted (13). In the transposed copy, the 3.7-kb fragment should then be reduced to 2.2 kb, whereas the 3.0-kb fragment remains unchanged. Indeed, as illustrated in Fig. 1, Southern blot analysis of the FDG^+ cell populations isolated by cell sorting (lanes p1, p2, and p3) revealed the expected 2.2-kb fragment upon hybridization with an nlsLacZ probe. Furthermore, densitometric analysis of the autoradiograms, taking the intensity of the 3.7-kb fragment as unity, indicated that the intensity of the newly generated 2.2-kb fragment was close to unity (namely, 1.0 ± 0.15 for three independent experiments, with a reference value of 1.1 ± 0.1 for clones c1 to c3 below), strongly suggesting that virtually all of the FDG^+ cells sorted by FACS acquired an additional copy in which the nlsLacZ^{RT} intron was precisely removed by splicing (accordingly, the relative intensity of the 3.0-kb fragment doubled, from 0.8 ± 0.2 to 1.7 ± 0.2). As expected too, hybridization of the blot with a *tk* probe (Fig. 1C) revealed the expected 900-bp *Sac*I fragment extending from the splice junction to the 3′ LTR in a retrotransposed copy.

These quantitative results were confirmed by Southern blot analysis of subclones randomly derived from the FDG^+ cell population by limiting dilution (lanes c1 to c4). Restriction of their genomic DNA by *Bam*HI, whose restriction site is unique in the transfected proviral copy and then directly yields the proviral copy number, disclosed at least one additional copy for all of the FDG^+ clones tested, without exception (Fig. 1D, lanes c1 to c4), and even two for clone c4. As expected, restriction of their DNA by *Sac*I disclosed a pattern identical to that of the original cell population in all cases (but one [see below]) upon hybridization with either the nlsLacZ (Fig. 1B) or the *tk* (Fig. 1C) probe. For one clone (clone c3), the 900-bp *tk* fragment and the 2.2-kb nlsLacZ fragment were actually observed, as expected for precise splicing upon retrotransposition, but the other expected nlsLacZ-marked fragment had an unexpected size (~5 kb instead of 3.0 kb), consistent with alteration of the transposed copy in its 5′ part. Similar results, i.e., splicing out of the intronic domain of the indicator gene, were also obtained with cell populations sorted by FACS from clones A-3 and A-4 (data not shown).

Retrotransposition of the marked provirus can occur at a high frequency. The absolute transposition frequency cannot be simply equated with the instantaneous proportion of β-gal^+ cells in a clonal cell population; accordingly, the frequency at which the marked provirus generates β-gal^+ variants was precisely measured by a method derived from that developed by Newcombe for procaryotes (21) and whose rationale is to measure accurately the fractional increase of β-gal^+ cells in a population as a function of time (Fig. 3). The clonality of β-gal^+ variant-producing clone A-2 was ascertained by subcloning (clone A-2.1), and two independent experiments (described in Fig. 3) allowed derivation of the absolute frequency at which the clonal cell population generates β-gal^+ variants; it was $2.7 \pm 0.9 \times 10^{-4}$ event per cell per generation. This value allows determination of the absolute transposition frequency of the marked provirus, taking into account that virtually all of the cells which scored blue should have acquired a transposed copy, according to the quantitative analysis described in the preceding section. Transposition frequency should be dependent upon the splicing efficiency of the intron in the indicator gene, since retrotransposed copies in which the intronic domain has not been eliminated should not yield the expected β-gal^+ phenotype. Actually, Northern (RNA) blot analysis of A-2 cell RNA with an nlsLacZ probe (A cells gave a strictly identical pattern [data not shown]) disclosed two major transcripts of 7.0 and 6.4 kb (Fig. 4, lane 3; other minor bands were observed but not further characterized), which should correspond to the unspliced and spliced transcripts of the marked provirus, respectively, as intron removal is expected to reduce the full-length transcript by 630 bp; in addition, the spliced transcript should not be revealed with an intronic probe (pA), as observed for the 6.4-kb band (Fig. 4, lane 4). Densitometric analysis of the autoradiogram indicated that intron splicing efficiency was close to 80% and should not noticeably restrict the sensitivity of the indicator gene. Accordingly, the value which can be derived for the absolute transposition frequency of the marked provirus in clone A-2 is 3.3×10^{-4} per cell per generation and also per marked provirus copy, as the Southern blot in Fig. 1D disclosed a single integrated marked copy per cell in the initial A clone.

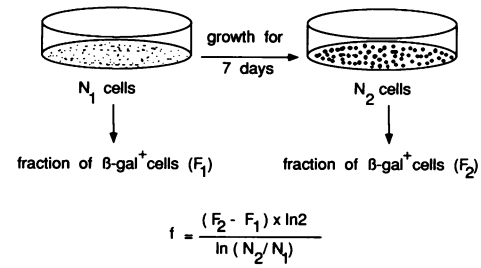

FIG. 3. Transposition frequency of the marked nlsLacZ^{RT} provirus. The protocol used for accurate measurement of the frequency of generation of X-Gal^+ cell variants among transcomplemented cells was a modification of the method of Newcombe (21); F_1 and F_2 and N_1 and N_2 are, respectively, the fractions of X-Gal^+ cells and the total numbers of cells at the beginning and end of the growth period. Cells from cultures in the exponential growth phase were plated in 10-cm-diameter dishes ($N_1 = 10^5$), grown for 7 days, and then trypsinized, pooled, and counted (N_2). F_1 and F_2 were measured upon X-Gal staining of a definite number of cells (5×10^3) in 24-well plates by counting the β-gal^+ cells under a microscope. The frequency of spontaneously arising X-Gal^+ cell variants (f [number of events per cell per generation]) was calculated from the equation shown, which was derived from equation 3 in reference 17.

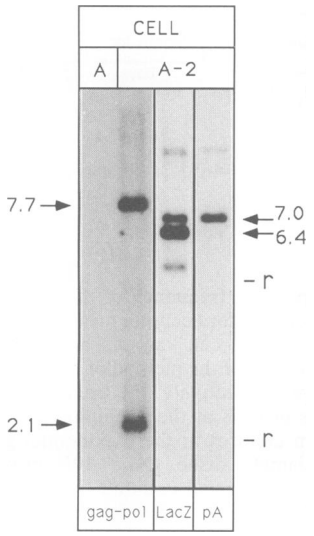

FIG. 4. Northern blot analysis of the proviral transcripts in *gag-pol*-transcomplemented cells (clone A-2) and control cells (clone A). Blots of poly(A)$^+$ RNAs from A and A-2 cells were hybridized with different ^{32}P-labelled probes, i.e., the fragment indicated by a bar in Fig. 1A for *gag-pol* transcripts, nlsLacZ, and pA. The spliced (6.4-kb) and unspliced (7.0-kb) proviral transcripts from the marked provirus and those from the transcomplementing *gag-pol*-containing structure (full-length 7.7-kb and subgenomic 2.1-kb proviral transcripts) are indicated by arrows. Their sizes were deduced from the relative positions of the murine 28S and 18S rRNAs (r). The percentage of spliced viral transcript for the marked provirus, as determined by scanning of the autoradiogram, was close to 80% (two Northern blots were analyzed).

Other transcomplemented clones were similarly analyzed (see the following sections and Fig. 5) and gave frequencies ranging from 3×10^{-6} to 3×10^{-4}.

Cell supernatants display RTase-containing particles but cannot confer the β-gal$^+$ phenotype. Induction of transposition of the marked provirus by the *gag-pol* gene is concomitant with the release of large amounts of RTase activity in the supernatant of the transcomplemented cells. RTase activity in the supernatant of clone A-2 (or of the A-2.1 subclone) was detected at a level roughly similar to that of a Ψ2 producer cell line ($E_{40}\Psi_B$ [25], which contains an MoMLV recombinant provirus), whose titer was close to 10^6 CFU/ml. The values obtained for three kinetic experiments, under conditions in which the counts per minute incorporated were linear with time, were 2,970 ± 350 and 1,085 ± 90 cpm/min for A-2 and $E_{40}\Psi_B$, respectively; no RTase activity (<10 cpm/min, i.e., the background level of the assay) was found in the supernatant of G355-5 cells containing the marked defective provirus alone (clone A). RTase activity is associated with viruslike particles, which were pelleted from A-2 cell culture supernatants and observed by electron microscopy (data not shown); these particles were approximately 0.1 μm in diameter with a heavily labeled internal core, as observed for type C particles and as previously characterized (28).

Despite the large number of retroviruslike particles released by A-2 or A-2.1 cells (see also the next section and Fig. 5 for other *gag-pol*-transcomplemented clones), the particle-containing supernatant, as expected, could not confer the β-gal$^+$ phenotype on feline G355.5 test cells (as evidenced by X-Gal staining) under conditions commonly used to test infectivity: large volumes of supernatant collected from A-2 cells (or from subclone A-2.1 cells) close to confluency were repeatedly tested (50 to 100 ml for cells maintained in culture for up to 6 months), and negative results were also obtained when similar volumes of supernatant were tested in the presence of Polybrene, which increases the titer of many infectious retroviruses at least 20-fold (26, 30). Accordingly, the viral titer of the supernatant, if any, should be less than 0.01 U/ml, a value at least 5,000-fold lower than the minimal value which would be required to account for the high-frequency generation of β-gal$^+$ variants by infectious particles (see the calculation in the Appendix; also see the Discussion).

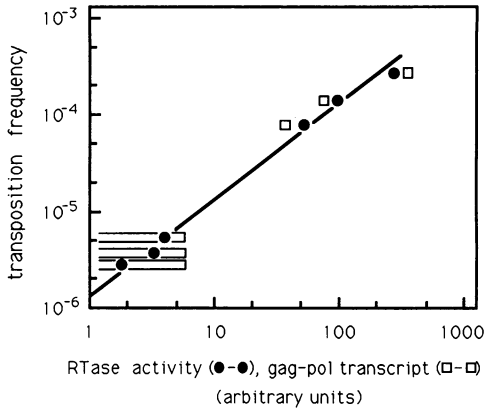

FIG. 5. Correlation between transposition frequency, RTase activity released in the supernatant, and levels of *gag-pol* transcripts for six transcomplemented clones. The solid line has a slope of unity; clone A-2 is included and corresponds to that with the highest transposition frequency; for three clones, only the upper limits for levels of *gag-pol* transcripts could be derived (horizontal bars).

Retrotransposition frequency of the marked provirus depends on the level of expression of the transfected *gag-pol* gene. A quantitative analysis of the transposition frequency, the level of RTase activity released in the supernatants, and the level of *gag-pol* gene expression was performed for six transcomplemented clones derived from clone A (including clone A-2 described above). As illustrated in Fig. 5, there was a linear relationship, over a range of 3 orders of magnitude, between the transposition frequency and the level of *gag-pol* gene expression measured as the amount of RTase activity released in the cell supernatant. Furthermore, the *gag-pol* gene transcripts in the transcomplemented clones were detected upon Northern blot analysis (Fig. 4, lane 2, shows clone A-2, and lane 1 shows clone A [control]), and their abundance was quantitated by densitometric scanning of the autoradiogram. As illustrated in Fig. 5, there was a positive correlation between the level of *gag-pol* gene transcripts, RTase activity, and the transposition frequency. We verified that the levels of marked-provirus transcripts (analyzed by Northern blot) were identical for all of the clones (data not shown) and thus were not responsible for the large variations in transposition frequency among the clones.

DISCUSSION

Indicator for in situ detection of retrotransposition. We constructed and characterized a novel indicator gene of retrotransposition, nlsLacZ^{RT}, which is adapted to in vivo studies. Its expression is triggered upon retrotransposition of the marked mobile element in which it is inserted and thus

confers a β-gal$^+$ phenotype on cells which can be unambiguously identified upon X-Gal or FDG staining. Actually, the nlsLacZ^{RT} gene inserted into an MoMLV provirus deleted for the three viral *gag*, *pol*, and *env* ORFs, when transfected into feline cells, does not spontaneously confer the β-gal$^+$ phenotype: upon X-Gal staining of more than 10^7 cells containing the defective marked retrotransposon, no β-gal$^+$ cells were detected. On the other hand, induction of transposition of the marked provirus upon transcomplementation with the MoMLV *gag-pol* gene results in generation of β-gal$^+$ cell variants at high frequency. Southern blot analysis of the genomic DNA of β-gal$^+$ cell populations sorted by FACS and of derived subclones demonstrated that the switch to the β-gal$^+$ phenotype was, in all cases, quantitatively correlated with the acquisition of at least one additional retrotransposed copy of the marked provirus in which the intron of the indicator gene had been precisely removed.

High-frequency transposition of defective retroviruses. An interesting outcome of this work was the spontaneous rate of intracellular transposition of the marked provirus which, upon transcomplementation with the *gag-pol* gene, can occur at a frequency as high as $(3 \pm 1) \times 10^{-4}$ event per cell per generation per marked provirus copy. Moreover, this value might still underestimate the actual retrotransposition frequency of the defective marked provirus, as transpositions into "silenced" regions of the genome might not be detected by the X-Gal staining assay (or the FDG staining assay, as their efficiencies were found to be identical). The observation of a cell clone (clone c4 in Fig. 1) containing two transposed copies of the marked provirus (1 clone over 6 randomly selected β-gal$^+$ clones analyzed, whereas for independent events the probability should be 1 clone per 100 to 1,000 β-gal$^+$ clones) suggests that intracellular retrotransposition might be a cooperative phenomenon and, as such, occur in bursts, as observed for drosophila retrotransposons (9, 18; reviewed in reference 7). Transcriptionally active genome regions may actually be preferred targets for retrotransposon integrations in the same way as previously described for infectious retroviruses (19, 27), resulting in "en cascade" transpositions and enhanced mutagenesis. In any case, the high-frequency transposition reached under these conditions and the experimental model that has been developed (more than 5% of the cells should score blue after a few months of culture) might be particularly adapted to analysis of the effect of an insertional mutagen in cells in culture and possibly also to the characterization of defined genes by transposon tagging.

Role of the *gag-pol* gene products. Interestingly, we demonstrated that over a 3-order-of-magnitude range there is a linear relationship between the transposition frequency of the marked nlsLacZ^{RT} provirus and the level of *gag-pol* gene expression measured by either Northern blot analysis of the *gag-pol* transcripts or the amount of RTase activity released in the cell supernatant. This correlation is further supported by our previous observations with other cell types, such as murine 3T3 and human HeLa cells (13, 28; unpublished data [see below]), in which transposition and RTase activity could be detected both at levels, respectively, about 100 times lower than (13, 28) or identical to (unpublished data) that reported for clone A-2.

RTase activity released by *gag-pol*-complemented cells, in amounts which may be as large as those encountered in the supernatant of a Ψ2 or wild-type MoMLV producer cell line, is associated with retroviruslike particles. This observation is in agreement with previous reports indicating that the viral *gag-pol* gene, and in several instances the Gag polyprotein alone, is sufficient for production of retroviruslike particles for murine type C retroviruses and human immunodeficiency virus (28 and references therein). These viruslike particles cannot confer the β-gal$^+$ phenotype on feline cells under conditions classically used to test for infectivity (more than 100 ml of supernatant tested, either in the absence or in the presence of Polybrene), and a simple calculation, detailed in the Appendix, indicates that if they were responsible for the high-frequency generation of β-gal$^+$ cells, their titer should be at least 5,000 times higher than the maximal overestimate of 0.01 U/ml from the assay described above. Similar particles were observed in previous experiments with closely related proviral structures in murine and human cells, at a much lower level in reference 28, and at a similar level in an unpublished analysis in murine 3T3 cells segregating variants carrying a retrotransposed copy of a *neo*^{RT}-marked provirus at a high frequency upon complementation by the *gag-pol* gene (10^{-4} event per cell and generation, correlated with the release in the supernatant of RTase activity at levels as high as that observed with the A-2 feline cells); these particles were also shown to be noninfectious, and in those cases extensive coculture experiments with test cells (13, 28; unpublished data) demonstrated (i) that they could not be the intermediates of transposition, as described above, and further (ii) that neither cryptic extracellular particles nor direct cell-to-cell interactions could be involved in the strictly intracellular transposition process.

Altogether, these data strongly suggest that the amount of *gag-pol* gene products available within a cell should be the critical factor controlling the transposition frequency. Characterization of the corresponding intracellular structural intermediates involved in the retrotransposition process will probably be a difficult task, since on the basis of the calculation in the Appendix, only one retrotransposition event takes place when 10^5 particles are released in the supernatant.

Perspectives. The positive correlation between the intracellular retrotransposition phenomenon and the production and release of defective retroviral particles strongly suggests that intracellular retrotransposition is a general phenomenon which also occurs in cells infected by replication-competent retroviruses. Thus, infected cells could be submitted to permanent mutagenesis, even if they are refractory to reinfection, and intracellular retrotransposition could be implicated in the generation of neoplasms which appear after a long latency period following acute retroviral infections. Preliminary experiments (28a) indicate that the marked defective provirus can also be complemented in *trans* for intracellular transposition by endogenous cellular elements, although at a much lower frequency, upon treatment of the cells with carcinogens such as 5-azacytidine or tumor promoters such as tetradecanoyl phorbol acetate. The nlsLacZ^{RT} indicator gene may, therefore, be a powerful tool for detection of such transposition events in situ, in particular in transgenic mice, and for causally linking them with tumor induction and/or progression.

APPENDIX

Calculation of the viral titer required to account for the generation of β-gal$^+$ variants among a clonal cell population producing extracellular intermediates of retrotransposition conferring the X-Gal phenotype. Formally, the number, dm, of β-gal$^+$ cells generated during an interval of time, dt, by a clonal cell population in the exponential growth phase, is given by the following equation: $dm = f \times N(t) \times dt$, where $N(t)$ is the number of cells in the culture and f is the frequency of generation of β-gal$^+$ variants per cell per unit of time (see equation 3 in reference 17, which applies independently of the underlying mechanism responsible for generation of the variants).

Then, if at time t_0 the supernatant was removed from the cells and tested for infectivity on G355.5 feline cells, the initial rate of gener-

ation of β-gal$^+$ variants among the test cells should be simply equal to $(dm/dt)t_0$, if one assumes that the test cells and the producer cells behave identically in response to infectious virus particles. Given a half-life for the viral particles of $t_{1/2}$, the measured titer, T, should be given by the following equation:

$$T = 1/v \int (dm/dt)t_0 \times exp(-t/t_{1/2}) \times dt = (1/v) \times f \times N(t_0) \times t_{1/2}$$

where v is the volume of the culture medium covering the producer cells.

The half-life, $t_{1/2}$, of the particles in the supernatant (which share closely related physical characteristics with type C retroviruses, despite the lack of an envelope protein) was derived from the half-life of their RTase-associated activity, measured as follows. Dishes (10-cm diameter, in triplicate) were inoculated with 10^6 A-2 cells in 10 ml of growth medium, and 2 days later, i.e., under steady-state conditions, the amount of RTase activity in the supernatant, $(RTase)_{eq}$, was measured; the dishes were then rinsed twice, covered with 10 ml of fresh medium, and incubated for various periods (up to 4 h) at 37°C; at various times (six measurements), supernatant samples were removed and their RTase activities were measured, thus yielding the initial rate of viral particle formation, $(dRTase/dt)t_0$; $t_{1/2}$ was then calculated as follows: $t_{1/2} = [\ln 2 \times (RTase)_{eq}]/(dRTase/dt)t_0$. A value of 8 ± 1 h was obtained, which is close to previous estimates of $t_{1/2}$ for infectivity of type C retroviruses, and therefore a posteriori confirms the equation of the $t_{1/2}$ for RTase activity with that for functionality. Accordingly, a value of approximately 50 X-Gal-conferring particles per ml can be derived for T [$N(t_0) = 7 \times 10^6$, $v = 10$ ml, $t_{1/2} = 8$ h, $f = 1.0 \times 10^{-5}$ events per cell per hour (2.7×10^{-4} events per cell per generation)].

ACKNOWLEDGMENTS

We specially acknowledge Z. Mishal and M. Lipinski for the cell sorting experiments. We thank C. Lavialle for critical reading of the manuscript, J.-F. Nicolas and C. Bonnerot for the gift of plasmid pGEM-nls-lacZ and invaluable suggestions and encouragement, and C. Paoletti and R. Monier for support.

This work was financed by the Centre National de la Recherche Scientifique (Unité associée 147), the Institut National de la Santé et de la Recherche Médicale (Unité 140), and the Association pour la Recherche sur le Cancer (contract 6552, awarded to T.H.).

REFERENCES

1. **Armentano, D., S. F. Yu, P. W. Kantoff, T. von Ruden, W. F. Anderson, and E. Gilboa.** 1987. Effect of internal viral sequences on the utility of retroviral vectors. J. Virol. **61**:1647–1650.
2. **Bender, M. A., T. D. Palmer, R. E. Gelinas, and A. D. Miller.** 1987. Evidence that the packaging signal of Moloney murine leukemia virus extends into the *gag* region. J. Virol. **61**:1639–1646.
3. **Boeke, J. D.** 1989. Transposable elements in *Saccharomyces cerevisiae*, p. 335–374. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
4. **Bonnerot, C., D. Rocancourt, P. Briand, G. Grimber, and J.-F. Nicolas.** 1987. A β-galactosidase hybrid protein targeted to nuclei as a marker for developmental studies. Proc. Natl. Acad. Sci. USA **84**:6795–6799.
5. **de la Luna, S., I. Soria, D. Pulido, J. Ortin, and A. Jiménez.** 1988. Efficient transformation of mammalian cells with constructs containing a puromycin-resistance marker. Gene **62**:121–126.
6. **Dührsen, U., J. Stahl, and N. M. Gough.** 1990. *In vivo* transformation of factor-dependent hemopoietic cells: role of intracisternal A-particle transposition for growth factor gene activation. EMBO J. **9**:1087–1096.
7. **Echalier, G.** 1989. Drosophila retrotransposons: interactions with genome. Adv. Virus Res. **36**:33–105.
8. **Gallagher, R. E., and R. C. Gallo.** 1975. Type C RNA tumor virus isolated from cultured acute myelogenous leukemia cells. Science **187**:350–353.
9. **Gerasimova, T. I., L. J. Mizrokhi, and G. P. Georgiev.** 1984. Transposition bursts in genetically unstable Drosophila melanogaster. Nature (London) **309**:714–716.
10. **Harbers, K., A. Schnieke, H. Stuhlmann, D. Jähner, and R. Jaenish.** 1981. DNA methylation and gene expression: endogenous retroviral genome becomes infectious after molecular cloning. Proc. Natl. Acad. Sci. USA **78**:7609–7613.
11. **Heberlein, C. M. Kawai, M.-J. Franz, G. Beck-Engeser, C. P. Daniel, W. Ostertag, and C. Stocking.** 1990. Retrotransposons as mutagens in the induction of growth autonomy in hematopoietic cells. Oncogene **5**:1799–1807.
12. **Heidmann, O., and T. Heidmann.** 1991. Retrotransposition of a mouse IAP sequence tagged with an indicator gene. Cell **64**:159–170.

12a. **Heidmann, T.** Unpublished data.

13. **Heidmann, T., O. Heidmann, and J. F. Nicolas.** 1988. An indicator gene to demonstrate intracellular transposition of defective retroviruses. Proc. Natl. Acad. Sci. USA **85**:2219–2223.
14. **Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith.** 1984. A short amino acid sequence able to specify nuclear location. Cell **39**:499–509.
15. **Kuff, E. L.** 1990. Intracisternal A particles in mouse neoplasia. Cancer Cells **2**:398–400.
16. **Kuff, E. L., and K. K. Lueders.** 1988. The intracisternal A-particle gene family: structure and functional aspects. Adv. Cancer Res. **51**:183–276.
17. **Luria, S. E., and M. Delbrück.** 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**:491–511.
18. **Mével-Ninio, M., M.-C. Mariol, and M. Gans.** 1989. Mobilization of the gypsy and copia retrotransposons in Drosophila melanogaster induces reversion of the ovo^D dominant female-sterile mutations: molecular analysis of revertant alleles. EMBO J. **8**:1549–1558.
19. **Mooslehner, K., U. Karls, and K. Harbers.** 1990. Retroviral integration sites in transgenic Mov mice frequently map in the vicinity of transcribed DNA regions. J. Virol. **64**:3056–3058.
20. **Mulligan, R. C., and P. Berg.** 1981. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. USA **78**:2072–2076.
21. **Newcombe, H. B.** 1948. Delayed phenotypic expression of spontaneous mutations in Escherichia coli. Genetics **33**:447–476.
22. **Nicolas, J.-F., and P. Berg.** 1983. Regulation of expression of genes transduced into embryonal carcinoma cells, p. 469–485. *In* L. M. Silver, G. R. Martin, and S. Strickland (ed.), Teratocarcinoma stem cells. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Nolan, G. P., S. Fiering, J. F. Nicolas, and L. A. Herzenberg.** 1988. Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β-D-galactosidase activity after transduction of Escherichia coli LacZ. Proc. Natl. Acad. Sci. USA **85**:2603–2607.
24. **Nusse, R.** 1986. The activation of the cellular oncogenes by retroviral insertion. Trends Genet. **2**:244–247.
25. **Rubenstein, J. L. R., J. F. Nicolas, and F. Jacob.** 1984. Construction of a retrovirus capable of transducing and expressing genes in multipotential embryonic cells. Proc. Natl. Acad. Sci. USA **81**:7137–7140.
26. **Sanes, J. R., J. L. R. Rubenstein, and J.-F. Nicolas.** 1986. Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. EMBO J. **5**:3133–3142.
27. **Sherdin, U., K. Rhodes, and M. Breindl.** 1989. Transcriptionally active genome regions are preferred targets for retrovirus integration. J. Virol. **64**:907–912.
28. **Tchenio, T., and T. Heidmann.** 1991. Defective retroviruses can disperse in the human genome by intracellular transposition. J. Virol. **65**:2113–2118.

28a. **Tchenio, T., et al.** Unpublished data.

29. **Teich, N., J. Wyke, T. Mak, A. Bernstein, and W. Hardy.** 1984. Pathogenesis of retrovirus-induced disease, p. 785–998. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Toyoshima, K., and P. K. Vogt.** 1969. Enhancement and inhibition of avian sarcoma viruses by polycations and polyanions. Virology **38**:414–426.