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Defective Retroviruses Can Disperse in the Human Genome by Intracellular Transposition

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Using an assay for retrotransposition detection (T. Heidmann, O. Heidmann, and J. F. Nicolas, Proc. Natl. Acad. Sci. USA 85:2219–2223, 1988), we demonstrated that a defective retrovirus deleted for the *gag*, *pol*, and *env* open reading frames can disperse in the genome of human HeLa cells by intracellular transposition, at a frequency close to 10^{-6} events per cell per generation. Transposition requires cooperation in *trans* for the *gag* and *pol* gene products and may be associated with the release of low amounts of noninfectious retroviruslike particles which are the hallmarks but not the intermediates of this transposition process. Similar events could account for the dispersion at high copy number of some of the human endogenous sequences related to retroviruses and for the occurrence of noninfectious retroviruslike particles in human placenta and several tumor cell lines (reviewed by E. Larsson, N. Kato, and M. Cohen, Curr. Top. Microbiol. Immunol. 148:115–132, 1989).

A defective provirus was constructed from a cloned murine leukemia retrovirus of ecotropic host range (Moloney murine leukemia virus [Mo-MuLV]) by deleting 6 kb of internal sequence encompassing the three *gag*, *pol*, and *env* open reading frames and introducing a frameshift 3′ to the initiation codon of the remaining *gag* fragment. This minimal proviral structure was marked with the previously described indicator gene for retrotransposition, *neo*(RT) (12), inserted in place of the deletion and slightly modified as indicated in Fig. 1. *neo*(RT) is engineered in such a way that in its initial configuration the neomycin gene, which codes for resistance to G418 in mammalian cells, is silent but should be activated upon transposition of the provirus through an RNA intermediate, which removes upon splicing a polyadenylation sequence blocking *neo* expression (Fig. 1A). The cells in which transposition has occurred should then be selected in G418 medium.

We introduced the marked defective provirus into human HeLa cells by cotransfection with pSV2gpt (18). Three HeLa clones were selected which contained at least one full-length copy of the transfected provirus, as evidenced by a complementation assay similar to that described in reference 12. In this assay, infection of the transfected HeLa cells by an amphotropic MuLV resulted in the production of viral particles conferring G418 resistance to test cells. These clones were further expanded and submitted to G418 selection. No spontaneous retrotransposition of the marked provirus could be detected (only one $G418^r$ variant was obtained for more than 5×10^7 cells in selection, which did not contain additional proviral copies but simply disclosed rearrangements in the indicator gene [data not shown]). We therefore attempted to complement in *trans* the defective structure for the *gag* and *pol* gene products. A plasmid containing the Mo-MuLV *gag* and *pol* genes under the genuine long terminal repeat (LTR)-contained promoter and polyadenylation signal (Fig. 1A) was introduced into the HeLa cell clones by cotransfection with a plasmid coding for resistance to hygromycin (pHyg [24]). Twelve hygromycin-resistant clones were derived from one of the initial clones (H4). After expansion and G418 selection, five of these clones segregated $G418^r$ variants, at an average apparent frequency of one cell per 10^6 cells. Southern blot analysis with a *neo* probe (neo in Fig. 1B) after genomic DNA restriction with an enzyme which cuts only once in the *neo*(RT)-marked defective provirus in all cases revealed an additional *neo*-containing proviral copy in the $G418^r$ clones (Fig. 1C). To demonstrate retrotransposition, we took advantage of the generation of a restriction site for *Sac*I at the splice junction if the intron of the indicator gene is correctly spliced (12). Southern blot analysis of *Sac*I-restricted genomic DNA from $G418^r$ clones with a thymidine kinase gene promoter probe (tk in Fig. 1B) revealed for all of them (15 of 15 $G418^r$ clones analyzed) the expected 900-bp band corresponding to the splice-3′ LTR fragment (Fig. 1E). Hybridization with a *neo* probe also revealed the expected 2.7-kb band corresponding to the 5′ LTR-splice fragment in the retrotransposed copy, but not in all cases (see, for instance, clone B-2 in Fig. 1D). This suggests, as reported in *Saccharomyces cerevisiae* for the Ty1 retrotransposon (3, 27) and further supported in the present case by enzyme restriction mapping (data not shown), that retrotransposition can be mutagenic for the provirus itself.

A precise determination of the frequency of retrotransposition was performed for one of the transcomplemented clones by fluctuation analysis, as described for prokaryotes by Luria and Delbrück (17) and illustrated in Fig. 2. An absolute frequency for the generation of $G418^r$ variants of $(1.5 \pm 0.4) \times 10^{-6}$ events per cell per generation was derived. Taking into account (i) that all $G418^r$ clones analyzed were associated with a retrotransposition event (see above and legend to Fig. 2A) and (ii) that the splicing efficiency of the engineered intron is close to 0.75 (see Northern [RNA] blot analysis of the proviral transcripts in Fig. 2B), the frequency of transposition of the marked provirus (corrected for "silent" retrotranspositions from unspliced proviral transcripts) should be 2×10^{-6} per cell per generation.

Direct involvement of the *gag* and *pol* gene products in the triggering of retrotransposition was established as follows.

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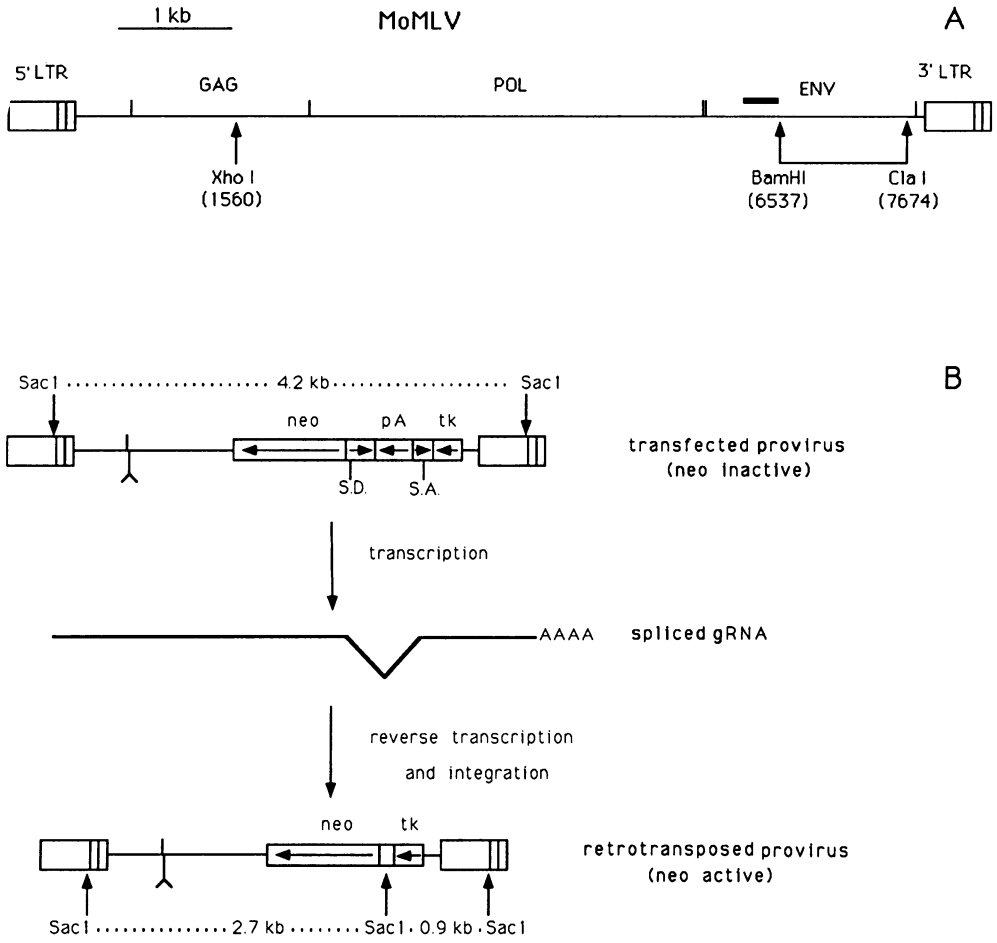
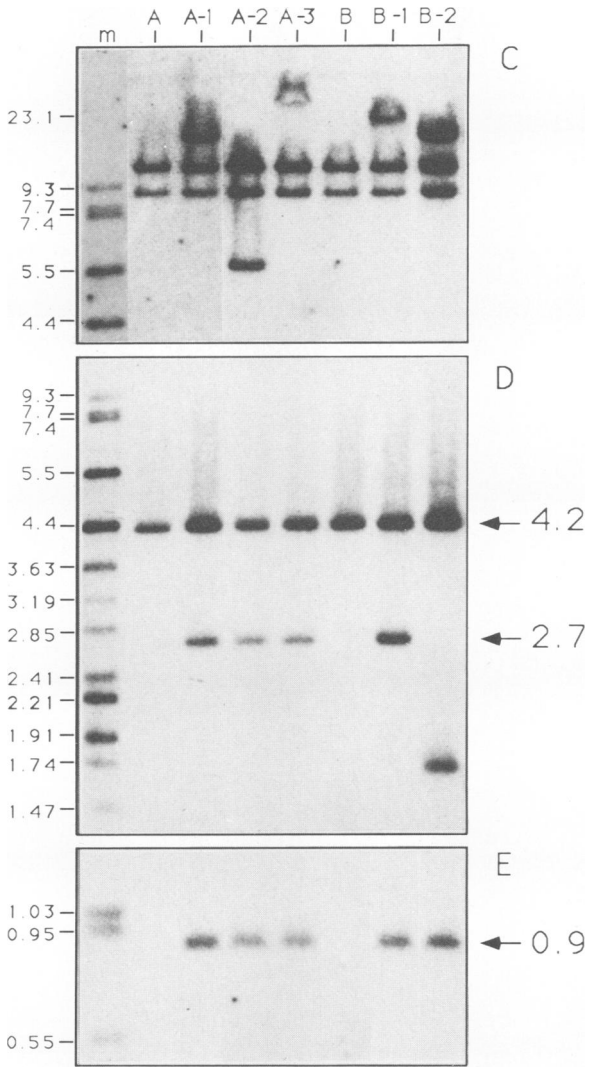


FIG. 1. Retroviral structures and retrotransposition detection. (A) Wild-type Mo-MuLV provirus (pMov3 [11]) with *gag*, *pol*, and *env* open reading frames. Restriction sites used for the construction of the defective marked provirus (see below) and the provirus for *gag-pol* transcomplementation (*Bam*HI-*Cla*I deletion) are indicated. Numbers refer to the distance from the viral RNA cap site. The heavy bar corresponds to the probe used in Fig. 3A. (B) Structure of the marked provirus and principle of the assay. *neo*(RT) is as described in reference 12 except for the splice donor-containing fragment, which was reduced in size upon *Mbo*I restriction. The arrows indicate the orientation of each genetic element. The neomycin-coding sequence (*neo*) under the control of the thymidine kinase promoter (*tk*) is inactive in the initial configuration because of an inserted sequence containing a polyadenylation signal (pA); pA is bracketed by splice donor and acceptor sites (S.D., S.A.). Upon transcription of the provirus, the engineered intron of the indicator gene should be removed, and in the transposed copy *neo* directly under the control of the *tk* promoter should be active. *neo*(RT) (a blunt-ended *Hin*dIII-*Ava*I fragment) was inserted in place of the deleted *Xho*I (1560) to *Cla*I (7674) domain of the wild-type Mo-MuLV (see panel A) after Klenow treatment. The 5′ part of the *gag* open reading frame was preserved because it contains *cis* sequences increasing the efficiency of viral genomic RNA encapsidation (1, 2), but it was inactivated by the introduction of a frameshift (indicated as ⅄, a 8-bp *Sac*II linker at position 623, initial construct by S. Goff). (C–E) Southern blot analysis of the DNAs of two H4 clones transcomplemented with *gag-pol* (clones A and B) and of G418^r variants (clones A-1 to A-3 and B-1 and B-2). (C) Analysis of the number of *neo*(RT)-marked provirus copies upon *Bam*HI restriction (which cuts once in pA), and hybridization with a *neo*-labeled probe. (D and E) Analysis of the structure of the additional marked provirus copies upon *Sac*I restriction and hybridization with a *neo* (D)- or *tk* (E)-labeled probe. The expected 4.2-kb, 2.7-kb, and 900-bp *Sac*I fragments are indicated by arrows; lane m, size markers (shown by numbers on left in kilobases). HeLa cells were grown in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum (GIBCO), 100 μg of streptomycin per ml, and 100 U of penicillin per ml at 37°C in 5% CO_2. The marked provirus was cotransfected with pSV2gpt (18) by the calcium phosphate method, and clones were selected in the presence of mycophenolic acid (25 μg/ml) plus xanthine (250 μg/ml). The *gag-pol*-containing plasmid was cotransfected with pHyg (24), and clones were selected in the presence of hygromycin (200 μg/ml; Sigma). G418^r cells were selected with 700 μg of G418 (GIBCO) per ml. Southern blotting and hybridization were done as described in reference 6, with ^{32}P-labeled random-primed DNA fragments.



(i) No G418^r variants could be isolated from 16 hygromycin-resistant clones obtained upon mock transfection of the H4 clone with the pHyg plasmid alone and subsequent hygromycin selection (up to 10^7 cells per clone in selection); (ii) all five clones which generated G418^r variants were shown a posteriori to contain several copies of the transfected *gag-pol* plasmid (data not shown), whose expression in the HeLa cells was demonstrated by Northern blot analysis, as shown for one of them in Fig. 3A; (iii) reverse transcriptase (RTase) activity was detected specifically in the supernatant of those clones which segregated G418^r variants. Levels of RTase activity ranged, depending on the clone, between 1 and 6% of that detected with a virus producer Ψ2 murine cell line as a positive control, whereas no activity was detected in the supernatant of HeLa cells or HeLa cells transfected with the pHyg plasmid alone (<0.1%, i.e., background level of the assay). Interestingly, analysis on sucrose density gradients of the cell culture supernatants (Fig. 3B) disclosed a peak of RTase activity at a density of 1.16 ± 0.05 g/ml, which corresponds to that of type C viral particles. Viruslike particles were actually detected in these fractions by electron microscopy (Fig. 3C). These results demonstrate functionality of the transfected Mo-MuLV-derived *gag-pol* sequences in the human cells. Moreover, they indicate, as suggested from previous reports for replication-defective retroviruses in murine and avian cells (19, 21, 22), and more recently demonstrated for human immunodeficiency virus *gag* or *gag-pol* gene constructs (9, 14, 23), that the *env* gene is not necessary for production of viruslike particles.

TABLE 1. Coculture experiments between cells producing G418^r variants (H4-B clone) and test cells (HeLa-*lacZ* cells) to demonstrate intracellular transposition of the marked provirus[a]

Expt	N_i	(LacZ$^+$/LacZ$^-$)$_i$	N_f	(LacZ$^+$/LacZ$^-$)$_f$	G418^r (LacZ$^+$/LacZ$^-$)
1	10^4	4	7.5×10^6	5.2	0/19
2	10^4	2	1.0×10^7	3.0	0/27
3	10^4	1	1.1×10^7	2.3	0/17

[a] N_i, N_f, Number of H4-B cells at the initial and final times of the cocultures, respectively, before G418 selection. (LacZ$^+$/LacZ$^-$)$_i$, (LacZ$^+$/LacZ$^-$)$_f$, number of HeLa-*lacZ* cells relative to H4-B cells in the cocultures in the same time interval. The number of G418^r clones obtained and their phenotype (LacZ$^+$/LacZ$^-$) are indicated for three independent experiments. HeLa-*lacZ* cells are a HeLa cell clone containing the nuclear location signal (nls)-*lacZ* reporter gene under LTR control (24a). Expression of the nls-*lacZ* gene (LacZ$^+$ phenotype) was detected by the presence of blue nuclei following X-gal staining (20).

The release of RTase activity and particles in the supernatant led us to investigate the intracellular character of the detected retrotransposition events. Although, as expected from the absence of *env* genes in all transfected plasmids, the supernatants were not infectious (i.e., cannot confer the G418^r phenotype to test cells under conditions used for infection), a definite demonstration was performed by coculture experiments between an H4 clone transcomplemented for *gag-pol* (H4-B) and test HeLa cells of identical origin but marked with a β-galactosidase *lacZ* gene. The rationale of the test is to assume that an extracellular intermediate of retrotransposition (whatever its nature) will target the different cell types with a probability equal to their relative proportion in the coculture. In three independent experiments, HeLa-*lacZ* cells were plated with a sufficiently small number of H4-B cells so that the probability of inoculating a G418^r variant, i.e., cells in which transposition had already occurred, was negligible (<0.01). The G418^r variants spontaneously arising during the period of growth of the coculture (1 to 2 weeks) were then recovered by G418 selection. As indicated in Table 1, all of them were *lacZ* negative.

In conclusion, intracellular transposition of a defective retroviral element can take place in human HeLa cells. This process does not require the envelope gene products and may be associated with the concomitant release of noninfectious viral particles. This situation could be related to the observation in human placenta (8, 13) and tumor cell lines (4, 5, 16) of rare retroviral particles containing RTase activity, which are most likely replication defective since viral envelope proteins were not detected by electron microscopy (4) and since attempts to propagate them as infectious retroviruses have failed (reviewed in references 15 and 25). As presently shown for an exogenous retrovirus, these particles could be the hallmarks of intracellular retrotransposition processes, responsible for the dispersion at high copy number of several retroviruslike elements in the human genome (references in 15 and 26). These results also suggest that many integrated proviral elements, originating either from an acute retroviral infection or from genetically transmitted endogenous elements, could cooperate to "cryptically" disperse in the genome and promote insertional mutagenesis by a series of intracellular retrotranspositions.

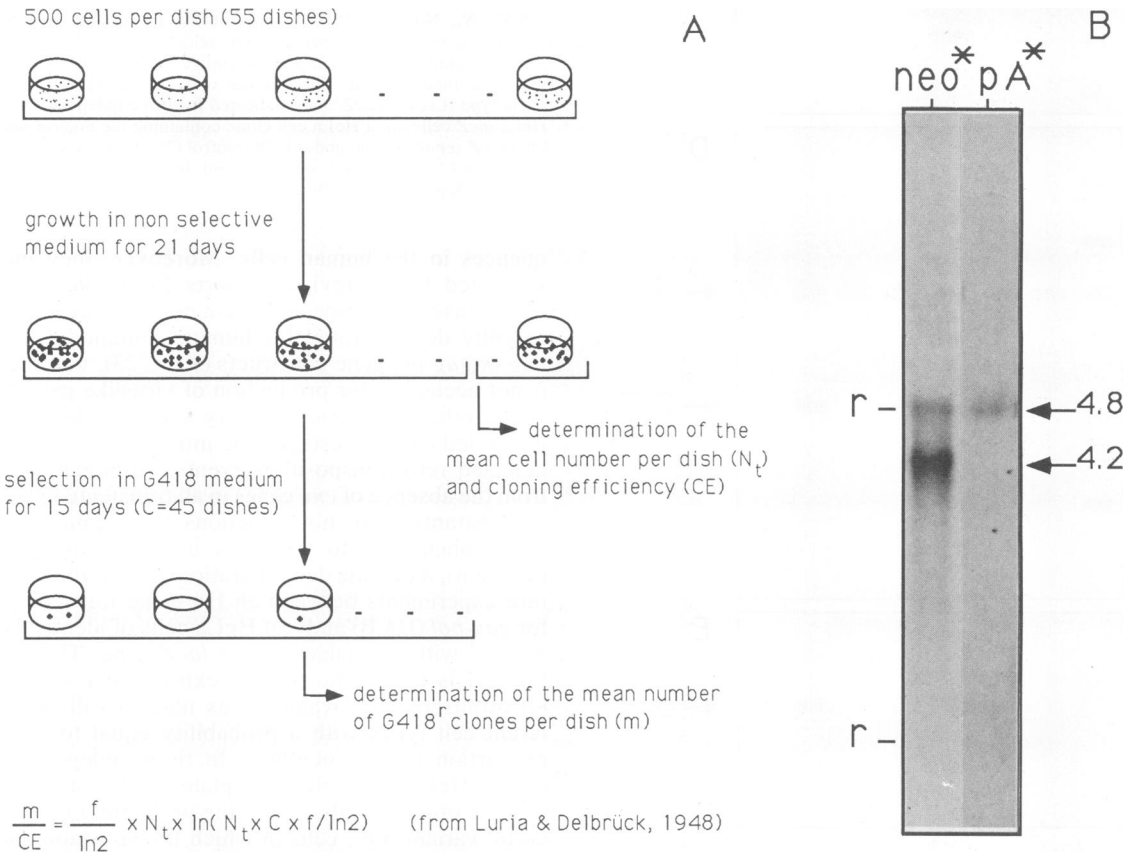


FIG. 2. Quantitative determination of the marked provirus transposition frequency. (A) Frequency of generation of G418^r variants measured by fluctuation analysis (17). Cells from clone H4-B were plated into 55 dishes (500 cells per dish) and grown in nonselective medium for 21 days. Five dishes were stained at day 21, and their colonies (if greater than 50 cells) were scored to determine cloning efficiency (CE). The 50 other dishes were individually trypsinized (0.5% trypsin, 0.5% EDTA in saline), and the cells were plated into new dishes allowing G418 selection (700 μg/ml). Five dishes were used to determine the average number of cells per dish (N_t) at the onset of the G418 selection. For the 45 dishes left (C), G418 selection was maintained for 15 days and then G418^r colonies were scored. In about one-half of the dishes containing G418^r cells, one clone was isolated and expanded and the DNA analysed by Southern blots; all of them (eight of eight) contained a retrotransposed provirus. The average number (m) of G418^r colonies per dish was best estimated from the percentage (p_0) of dishes containing no colony as $m = -\ln(p_0)$, since it minimizes errors due to clonal dispersion within individual dishes. The frequency of spontaneous generation of G418^r cells per cell per generation (f) corrected for cloning efficiency (CE = 0.19 ± 0.02) was computed for two independent experiments from equation 8 of reference 17 (given in the figure). (B) Northern blot characterization of the marked provirus transcripts and of intron splicing efficiency. Northern blots of total RNA from clone H4-B were hybridized with either a *neo*- or a pA (fig. 1)-radiolabeled probe. A major transcript of 4.2 kb was detected with the *neo* probe, which corresponds to the proviral transcript in which the indicator gene's intron is spliced as it is not lightened by the intronic pA probe, whereas the 600-bp-longer transcript, lightened by both probes, corresponds to the unspliced transcript. After scanning of the autoradiograms, a value for intron splicing efficiency of 0.75 ± 0.05 was derived. Total RNA was extracted by the guanidinium-cesium chloride procedure and electrophoresed in 1% agarose gel with formaldehyde and blotted as described in reference 7. Prehybridization, hybridization, and washing were as for Southern blots.

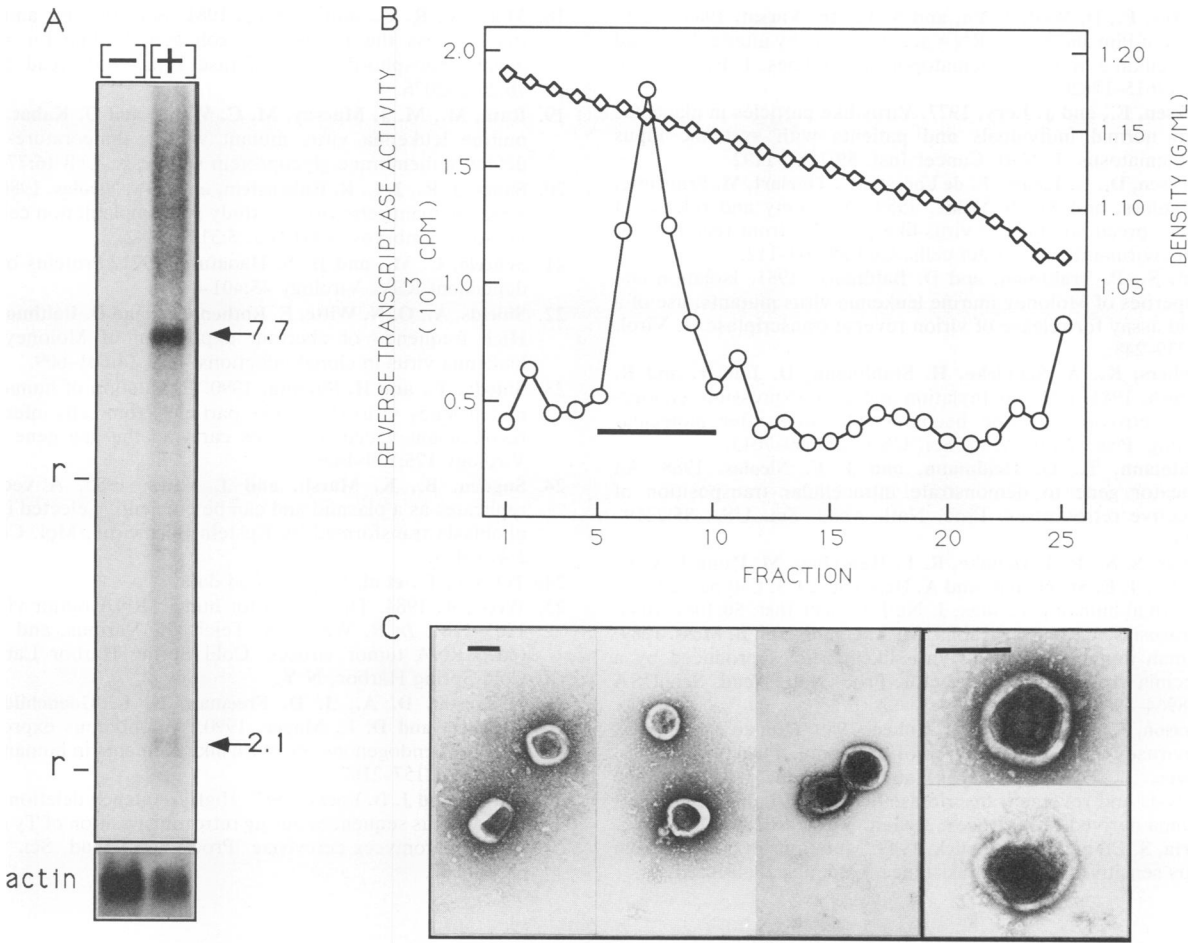


FIG. 3. Expression of the transfected murine *gag-pol* gene in stable HeLa cell clones. (A) The *gag-pol* gene under the control of the LTRs was derived from the cloned Mo-MuLV upon deletion of the *Bam*HI-*Cla*I envelope domain (Fig. 1A) and was introduced into the cells as indicated in the legend to Fig. 1. Total RNA from clone H4 (lane −) and from the transcomplemented clone H4-B (lane +) was analyzed by Northern blotting as described in the legend to Fig. 2. The DNA fragment used as a probe for viral mRNA is indicated in Fig. 1A with a heavy horizontal bar (bp 6236 to 6537). As a control, filters were hybridized with a murine β-actin probe. Two transcripts were detected (see arrows), a 7.7-kb mRNA corresponding to the full-length proviral transcript and a 2.1-kb mRNA corresponding to the subgenomic Mo-MuLV transcript (mouse 28S and 18S rRNAs are indicated as r); methods are as described in the legend to Fig. 2. (B and C) Characterization of released retroviruslike particles. (B) Determination of the buoyant density of particles containing RTase activity. Supernatants from H4-B cells were harvested (30 ml), filtered through 0.22-μm-pore-size filters, and concentrated by centrifugation (2 h in an SW28 rotor at 22,000 rpm, 4°C). The pellet was suspended in 200 μl of TNE (10 mM Tris HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA), layered on top of a 25 to 45% linear sucrose gradient in an SW41 rotor, and centrifuged for 12 h at 35,000 rpm at 4°C. Twenty-five fractions (~0.5 ml) were collected, and RTase enzymatic activity (○) was measured for 10 μl of each fraction as described in reference 10. (C) Electron microscopy of the negatively stained RTase-containing particles. Fractions corresponding to the peak of RTase were pooled (see bar in panel B), diluted in TNE, and centrifuged for 2 h in an SW41 rotor at 35,000 rpm at 4°C. The pellet was resuspended in 50 μl of TNE, and 5 μl was deposited on carbon-coated grids and stained with 1% uranyl acetate without prior fixation; the bars represent 0.1 μm.

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REFERENCES

1. **Armentano, D., S. 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., D. J. Garfinkel, C. A. Styles, and G. R. Fink.** 1985. Ty elements transpose through an RNA intermediate. Cell **40**:491–500.
4. **Boller, K., H. Frank, J. Löwer, R. Löwer, and R. Kurth.** 1983. Structural organisation of unique retrovirus-like particles budding from human teratocarcinoma cell lines. J. Gen. Virol. **64**:2549–2554.
5. **Bronson, D. L., E. E. Fraley, J. Fogh, and S. S. Kalter.** 1979. Induction of retroviral particles in human testicular tumor (Tera-1) cell cultures: an electron microscopic study. JNCI **63**:337–339.
6. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81**:1991–1995.

7. **Dautry, F., D. Weil, J. Yu, and A. Dautry-Varsat.** 1988. Regulation of Pim and Myb mRNA accumulation by interleukin 2 and interleukin 3 in murine hematopoietic cell lines. J. Biol. Chem. **263**:17615–17620.
8. **Dirksen, E., and J. Levy.** 1977. Virus-like particles in placentas from normal individuals and patients with systemic lupus erythematosus. J. Natl. Cancer Inst. **59**:1187–1192.
9. **Gheysen, D., E. Jacobs, F. de Foresta, C. Thiriart, M. Francotte, D. Thines, and M. de Wilde.** 1989. Assembly and release of HIV-1 precursor $Pr55^{gag}$ virus-like particles from recombinant baculovirus-infected insect cells. Cell **59**:103–112.
10. **Goff, S., P. Traktman, and D. Baltimore.** 1981. Isolation and properties of Moloney murine leukemia virus mutants: use of a rapid assay for release of virion reverse transcriptase. J. Virol. **38**:239–248.
11. **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.
12. **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.
13. **Kalter, S. S., R. J. Helmke, R. L. Heberling, M. Panigel, A. K. Fowler, J. E. Strickland, and A. Hellman.** 1973. C-type particles in normal human placentas. J. Natl. Cancer Inst. **50**:1081–1084.
14. **Karacostas, V., K. Nagashima, M. A. Gonda, and B. Moss.** 1989. Human immunodeficiency virus-like particles produced by a vaccinia virus expression vector. Proc. Natl. Acad. Sci. USA **86**:8964–8967.
15. **Larsson, E., N. Kato, and M. Cohen.** 1989. Human endogenous proviruses. Curr. Top. Microbiol. Immunol. **148**:115–132.
16. **Löwer, J., E. M. Wondrak, and R. Kurth.** 1987. Genome analysis and reverse transcriptase activity of human teratocarcinoma derived retroviruses. J. Gen. Virol. **68**:2807–2815.
17. **Luria, S. E., and M. Delbrück.** 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**:491–511.
18. **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.
19. **Ruta, M., M. J. Murray, M. C. Webb, and D. Kabat.** 1979. A murine leukemia virus mutant with a temperature-sensitive defect in membrane glycoprotein synthesis. Cell **16**:77–88.
20. **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.
21. **Scheele, C. M., and H. S. Hanafusa.** 1971. Proteins of helper-dependent RSV. Virology **45**:401–410.
22. **Shields, A., O. N. Witte, E. Rothenberg, and D. Baltimore.** 1978. High frequency of aberrant expression of Moloney murine leukemia virus in clonal infections. Cell **14**:601–609.
23. **Shioda, T., and H. Shibuta.** 1990. Production of human immunodeficiency virus (HIV)-like particles from cells infected with recombinant vaccinia viruses carrying the gag gene of HIV. Virology **175**:139–148.
24. **Sugden, B., K. Marsh, and J. Yates.** 1985. A vector that replicates as a plasmid and can be efficiently selected in B-lymphoblasts transformed by Epstein-Barr virus. Mol. Cell. Biol. **5**:410–413.

24a. **Tchenio, T., et al.** Unpublished data.

25. **Weiss, R.** 1984. The search for human RNA tumor viruses, p. 1205–1281. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Wilkinson, D. A., J. D. Freeman, N. L. Goodchild, C. A. Kelleher, and D. L. Mager.** 1990. Autonomous expression of RTVL-H endogenous retroviruslike elements in human cells. J. Virol. **64**:2157–2167.
27. **Xu, H., and J. D. Boeke.** 1987. High-frequency deletion between homologous sequences during retrotransposition of Ty elements in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA **84**: 8553–8557.