JOURNAL OF VIROLOGY, Mar. 1989, p. 1460–1464
0022-538X/89/031460-05$02.00/0
Copyright © 1989, American Society for Microbiology
Vol. 63, No. 3

Inhibition of RNase H Activity and Viral Replication by Single Mutations in the 3′ Region of Moloney Murine Leukemia Virus Reverse Transcriptase

ROY REPASKE,[1]* JANET W. HARTLEY,[2] MARK F. KAVLICK,[1] RAYMOND R. O'NEILL,[3] AND JOAN B. AUSTIN[2]

Laboratory of Molecular Microbiology[1] and Laboratory of Immunopathology,[2] National Institute of Allergy and Infectious Diseases, and Developmental and Metabolic Neurology Branch, National Institute of Neurological and Communicative Disorders and Stroke,[3] Bethesda, Maryland 20892

Received 1 August 1988/Accepted 29 November 1988

Selected conserved amino acids in the putative RNase H domain of reverse transcriptase (RT) were modified in a molecularly cloned infectious provirus and in a Moloney murine leukemia virus RT expression vector by site-directed mutagenesis. Substitution of either of two conserved aspartic acid residues in proviral DNA prevented production of infectious particles in transfected NIH 3T3 cells, and the same modifications depressed RT-associated RNase H activity by more than 25-fold with little or no effect on polymerase activity.

Reverse transcriptase (RT), encoded by a major portion of the retroviral *pol* gene, possesses three catalytic activities required for retroviral replication: RNA- and DNA-dependent DNA polymerase and RNase H (14, 36). The functional domain of RNA-dependent DNA polymerase has been assigned to the N-terminal region of RT on the basis of results for a variety of mutants (10, 11, 26, 32). Conserved retroviral amino acids have been identified in this region (16, 33), and these amino acids are found in certain DNA viruses which express RNA-dependent DNA polymerase activity (17, 31, 33) as well as in other genetic elements having putative RT polymerases (13, 34). The correlation of polymerase activity with these conserved sequences suggests a functional role for these residues.

Johnson et al. (8) proposed the C-terminal 160 amino acids of Moloney murine leukemia virus (M-MuLV) RT as the RNase H domain on the basis of extensive amino acid homology with *Escherichia coli* RNase H, although significantly fewer conserved sequences were found when alignments included other RTs. Tanese and Goff (32) provided experimental support for this proposal by demonstrating with a series of linker insertion mutants that M-MuLV polymerase and RNase H activities are associated with the N- and C-terminal regions, respectively, of RT. In an expanded sequence alignment of *E. coli* RNase H with many retroviral *pol* sequences (Fig. 1), eight invariant amino acid residues are present in all retroviral *pol* genes; of these, six residues (Fig. 1, stars) are common to both RTs and *E. coli* RNase H, and two (Fig. 1, carets) are unique to RT sequences. For reference, the positions of the conserved amino acids within the sequence are identified in Fig. 1. Relatedness of these dispersed conserved amino acids is supported by their relative positions in the sequence as well as by the context of their highly conserved flanking amino acids (Fig. 1, underlines). For example, in region A, the single conserved aspartic acid is usually flanked by Tyr or Phe and Thr or Ser on the N-terminal side and by Gly and Ser on the C-terminal side. On the assumption that conserved amino acids may be associated with important functional properties of the enzyme, certain amino acid residues in the A and C regions (Fig. 2) were modified by site-directed mutagenesis. Effects on M-MuLV replication and on polymerase and RNase H activities were evaluated.

Of the four amino acids modified (Fig. 2), two were conserved Asp residues. Mutants 8-9 and 116 contained base changes which encoded Gly instead of Asp in region A and Cys instead of Asp in region C, respectively (Fig. 1 and 2). Mutant 0-4 is a double mutant containing the mutation present in 8-9 and an Asn-to-Asp change in region C (Asn was conserved only in RT sequences [Fig. 1]). The fourth mutant, 46-4, has a Leu residue replacing the Thr which occurs in most RT sequences and in *E. coli* RNase H (region C). The 46-4 mutation was also back-mutated (Leu→Thr, designated 46-4R) to reestablish the normal sequence. M-1 was the control, with unmodified M-MuLV DNA sequences. Mutations were introduced into a cloned, circularly permutated infectious M-MuLV provirus that was originally isolated from a Hirt supernatant (7) and cloned into pBR322 at the *Sal*I site (provided by S. K. Chattopadhyay and cloned by C.-M. Wei). Mutagenesis was performed on a 1.5-kilobase *Sph*I segment (extending from the *Sph*I site in pBR322 to the same restriction site in *pol* of M-MuLV) subcloned into M13mp18. The method of Zoller and Smith (38) was used for oligonucleotide-directed mutagenesis with an appropriately mismatched synthetic oligodeoxyribonucleotide. The entire 1.5-kilobase mutated *Sph*I insert was sequenced (24) to confirm that other mutations had not occurred. Deleted M-MuLV pBR322 was reconstructed with the mutated *Sph*I fragment, *Sal*I restricted to release the provirus, and concatemerized for use in transfection.

Generation of infectious progeny virus by transfected NIH 3T3 cells was determined by the XC plaque assay (22). Transfection with each mutant DNA was performed with a confluent culture of NIH 3T3 cells by the $CaPO_4$ precipitation method (3). At 24 h, each transfected cell culture was rinsed with serum-free medium, trypsinized, and split. Representative results for syncytium-inducing activity in the XC plaque assay in one set of dishes are presented in Table 1, column 1. Infectious virus from transfection with parental Moloney M-1 (positive control) was readily detected. On the other hand, no XC plaques were found with transfected DNA from mutants 8-9 (four tests), 0-4 (six tests), or 116

* Corresponding author.

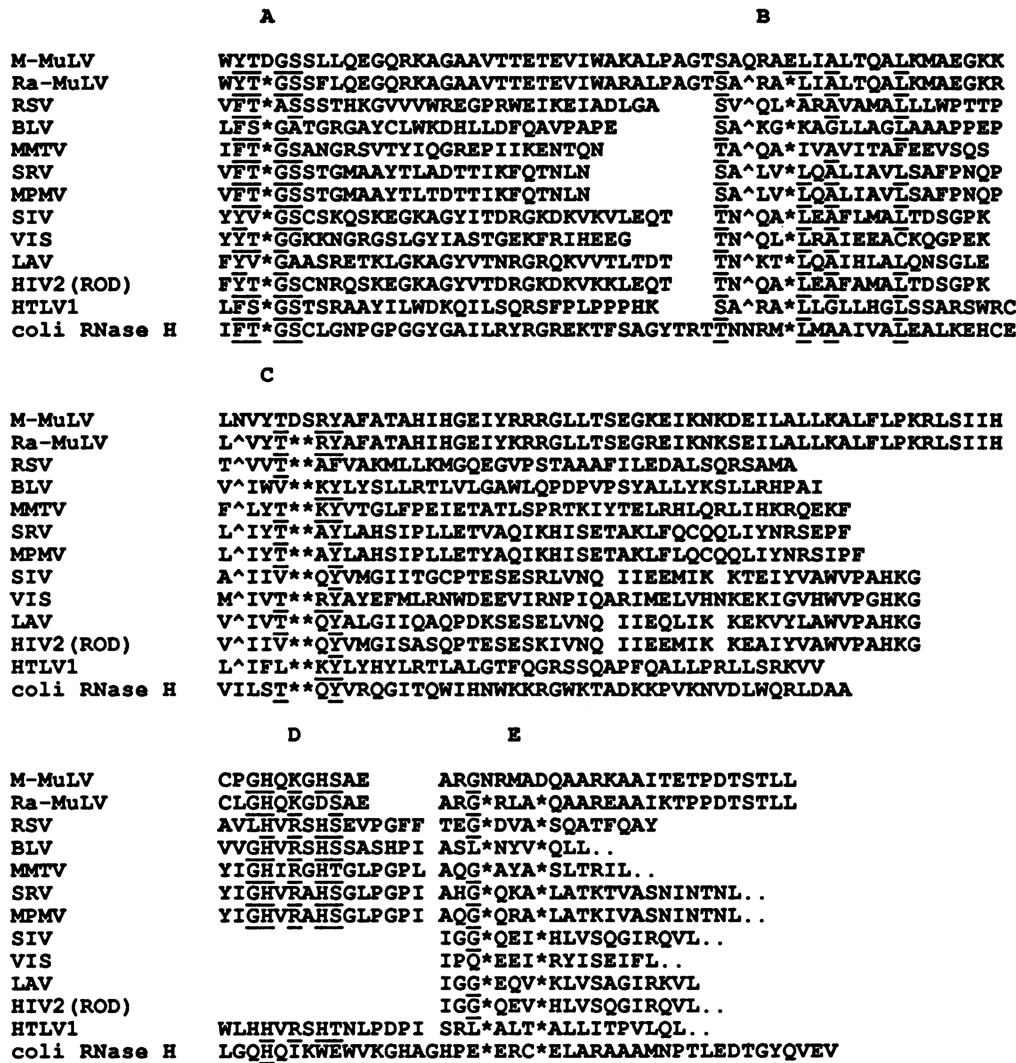

FIG. 1. Amino acid homology between C-terminal M-MuLV RT sequences, other retroviral *pol* sequences, and *E. coli* RNase H. Symbols indicate amino acids conserved in all sequences (★), conserved in all retroviral *pol* sequences but not in the *E. coli* RNase H sequence (^), and highly conserved (—). Letters A to E above the sequences indicate regions with clusters of homologous sequences. Sequences shown and nucleotide reference numbers corresponding to the first codon are as follows: M-MuLV, 1458 (28); radiation MuLV (Ra-MuLV), 4172 (12); Rous sarcoma virus (RSV), 3841 (25); bovine leukemia virus (BLV), 3628 (23); murine mammary tumor virus (MMTV), 1366 (2); simian retrovirus (SRV), 4602 (18); Mason-Pfizer monkey virus (MPMV), 4590 (30); simian immunodeficiency virus (SIV), 3652 (1); visna lentivirus (VIS), 3416 (29); lymphadenopathy-associated virus (LAV), 3413 (37); human immunodeficiency virus [HIV2(ROD)], 3695 (4); human T-cell leukemia virus (HTLV1), 3709 (27); and *E. coli* RNase H, 19 (9).

(one test) initially or after 2 to 10 subsequent cell culture transfers. The results of infectivity tests of supernatant fluids from NIH 3T3 or SC-1 (5) transfected cells were also negative (data not shown). Unlike the negative results obtained with these mutants, a small number of plaques were obtained with mutant 46-4. Mutant 46-4, which had been back-mutated to wild-type sequence (46-4R), gave a plaque count equal to that obtained with the M-1 control.

Rescue of replication-defective ecotropic M-MuLV may be achieved by superinfection with competent XC-negative helper viruses, such as amphotropic or mink cell focus-forming (MCF) MuLVs (19). XC-negative, infectious amphotropic MuLV (MuLV-A) was simultaneously added to transfected cells in the second dish of each set ($10^{4.4}$ mink S^+L^- focus-forming units of strain 1504A) (6) to evaluate the effect of a helper virus (Table 1). Under these conditions, virus was rescued from cells transfected with replication-defective mutants 8-9, 0-4, and 116. Rescue could also be effected by cotransfection with MCF MuLV proviral DNA (Table 1). Superinfection with MuLV-A produced no increase in the number of XC plaques obtained with M-1 or 46-4R transfected DNAs. In contrast, a 100-fold increase in the number of plaques resulted from superinfection of mutant 46-4. Amplification of XC plaques suggests that mutation 46-4 partially suppressed viral replication.

Virus rescued by superinfection with MuLV-A had phenotypic properties consistent with those of M-MuLV: interference assays showed that the virus expressed a functional ecotropic envelope (data not shown), and rescued virus also retained the NB tropism of the parental M-MuLV (data not shown).

The effect of the mutations on the function of polymerase

	A							C										
	4158							4329										
M-MuLV	TGG trp	TAC tyr	ACG thr	GAT asp	GGA gly	AGC ser	...(156 bp)...	CTA leu	AAT asn	GTT val	TAT tyr	ACT thr	GAT asp	AGC ser	CGT arg	TAT tyr	GCT ala
M-1	*	*	*	*	*	*		*	*	*	*	*	*	*	*	*	*
8-9	*	*	*	GGT gly	*	*		*	*	*	*	*	*	*	*	*	*
0-4	*	*	*	GGT gly	*	*		*	GAT asp	*	*	*	*	*	*	*	*
46-4	*	*	*	*	*	*		*	*	*	*	CTA leu	*	*	*	*	*
116	*	*	*	*	*	*		*	*	*	*	*	TGC cys	*	*	*	*

FIG. 2. Mutations introduced in the C-terminal RT region of M-MuLV by site-directed mutagenesis. The normal M-MuLV proviral sequence in the C-terminal region of RT, nucleotide sequence reference numbers, and deduced amino acids (28) are shown with the first entry. Mutant designations, nucleotide changes (underlined), and amino acid changes are indicated. Symbols and abbreviations: *, normal M-MuLV sequences in that position; A and C, regions corresponding to those shown in Fig. 1; M-1, control with normal M-MuLV sequences which were manipulated in the same way as mutant sequences; bp, base pairs.

and RNase H activities was assessed with cloned M-MuLV RT. For this purpose, vector pB6B15.23 (21), which expresses an enzymatically active RT fusion protein in *E. coli*, was used. Identical mutations described for proviral DNAs were introduced into the expression vector by replacing the *Bgl*II-*Sph*I fragment in the vector with the equivalent mutated fragment from M13 containing the mutation. Designations used for mutant RTs are the same as those used for proviral DNA. Before RT activities in *E. coli* extracts were determined, it was necessary to purify the RT fusion protein (DEAE-cellulose and phosphocellulose column [21]) to eliminate *E. coli*-associated DNA polymerase and RNase H activities. Protein concentrations of the partially purified enzymes were determined by direct densitometry of the RT protein bands on a 10% sodium dodecyl sulfate-polyacrylamide gel stained with Coomassie blue. Single 71-kilodalton reactive bands were identified by immunoblot analysis (35) with RT-specific polyclonal rabbit antibody.

Specific activities for incorporation of each ^{3}H-deoxynucleoside triphosphate [^{3}H]dNTP) with its respective homopolymer template-primer pair were determined, since mutation may have affected the polymerase activity of only one of the four nucleotide substrates. Reaction rates with poly(rI) $\cdot$ oligo(dC)$_{12-18}$ and poly(rC) $\cdot$ oligo(dG)$_{12-18}$ were increased by making a separate cocktail of preheated poly(rI) or oligo(dG) (90°C, 1 min) before the corresponding oligonucleotide or polynucleotide and dNTP were added. The reaction mixture (21) was modified by replacing NaCl with 55 mM KCl and increasing Nonidet P-40 to 0.09%. Polymerase activity was measured by the rate of incorporation of [^{3}H]dNTP in the trichloroacetic acid-insoluble fraction retained by nitrocellulose filters.

The rates of incorporation of each dNTP with mutant enzymes 8-9 and 0-4 are the same as the rates obtained with the unmodified control enzyme (M-1) and with the reversed mutant (46-4R) (Table 2). Similar values were also obtained with the highly purified M-MuLV RT (Bethesda Research Laboratories). These data show that mutant enzymes 8-9 and 0-4 have normal polymerase activities with each of the dNTPs. Consequently, mutations which caused proviral mutants 8-9 and 0-4 to be replication defective (Table 1) cannot be related to any change in polymerase activity. Enzymes 46-4 and 116 had RT activities with each dNTP substrate that were equivalent and about 50% lower than those of the normal control, enzyme M-1 (Table 2). Provirus 46-4 was capable of producing infectious particles without helper virus, and provirus 116 was not (Table 1). The decreased polymerase activity observed, therefore, could

TABLE 1. Detection of replication-defective M-MuLV mutants

Mutant (μg of DNA)	Log_{10} XC plaques per dish[a] after superinfection or cotransfection with:		
	No virus	MuLV-A	MCF MuLV DNA[b]
None	—[c], —*	—, —*	—*
M-1 (0.5)	3.0		
M-1 (0.1)	1.6	1.6	
8-9 (1.0)	—, —*	1.6	
0-4 (0.5)	—*	2.6*	3.6*
116 (0.5)	—	2.7	
46-4 (0.5)	1.0	2.9	
46-4R (0.5)	2.2	2.4	

[a] XC tests were performed 5 to 6 days after transfer of transfected NIH 3T3 cells. In some cases (*), 10^5 SC-1 cells were added to the assay plates in an attempt to amplify virus.

[b] Cultures were cotransfected with 0.2 μg of AKR13 MCF MuLV proviral DNA (provided by A. S. Khan).

[c] —, No plaques detectable.

TABLE 2. RT and RNase H activities of normal and mutant enzymes

Enzyme source	RT[a] sp act				RNase H sp act[b]
	dTTP	dCTP	dATP	dGTP	
BRL[c]	84,000	7,200	2,300	1,200	543
M-1	100,000	6,600	4,200	1,300	581
8-9	104,000	6,100	4,200	1,500	4
0-4	83,000	6,100	3,900	1,100	23
116	41,000	4,200	2,300	600	22
46-4	57,000	4,200	2,200	700	453
46-4R	97,000	8,000			540

[a] Assay for incorporation of the specific dNTP (0.019 mM) indicated. Each reaction was performed with the appropriate homopolymer polyribonucleotide-oligodeoxyribonucleotide template-primer pair. Values are expressed as picomoles of [^{3}H]dNTP incorporated per microgram of enzyme protein per 15 min.

[b] Picomoles of acid-soluble [^{3}H]AMP released per microgram of enzyme protein per 30 min.

[c] BRL, Bethesda Research Laboratories.

not by itself be a sufficient explanation for the replication defect in provirus 116.

RNase H activity was measured by the release of trichloroacetic acid-soluble [^{3}H]AMP from a [^{3}H]RNA-DNA M13 hybrid prepared with *E. coli* RNA polymerase (21). The assay was modified by the substitution of KCl for NaCl and the addition of 0.1% Nonidet P-40. Enzymes with normal M-MuLV sequences (M-1, 46-4R, and BRL RT) solubilized equal amounts of [^{3}H]AMP per microgram of enzyme protein in 30 min (Table 2). On the other hand, RNase H activities of mutant enzymes 8-9, 0-4, and 116 were reduced 25- to 130-fold. Inhibition may have been even greater, because counts of released [^{3}H]AMP just exceeded background counts. The additional mutation (Asn to Asp) in mutant 8-9 which produced 0-4 had no detectable influence on RNase H activity; its effect, if any, could not be evaluated. Mutation 46-4 had little effect on RNase H activity. Modification of two adjacent amino acids (Fig. 2, Thr in mutant 46-4 and Asp in mutant 116) produced dramatically different quantitative effects on RNase H activity. These results illustrate the amino acid specificity with regard to function.

A direct correlation between mutations which severely depressed RNase H activity and those which produced defective replication of virus has been shown. In the putative RNase H domain, substitution of either of two conserved Asp residues prevented productive infection of M-MuLV, apparently because of the restricted RNase H activity. Such a result would be anticipated in view of the functional role of RNase H in the retroviral life cycle (14, 15, 20), but it has not been directly demonstrated by a single codon sense change in an otherwise complete viral sequence. These data do not address the question of whether the aspartic acids are constituents of an active site or are required for conformational integrity of RNase H. However, they do identify two amino acids which could be targets for chemotherapeutic intervention in retroviral infections.

We thank S. P. Goff for generously providing vector pB6B15.23, S. K. Chattopadhyay for making available the infectious clone of M-MuLV, and A. S. Khan for the clone of AKR13 MCF MuLV.

LITERATURE CITED

1. **Chakrabarti, L., M. Guyader, M. Alizon, M. D. Daniel, R. C. Desrosiers, P. Tidlaus, and P. Sonigo.** 1987. Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. Nature (London) **328:** 543–547.
2. **Deen, K. C., and R. W. Sweet.** 1986. Murine mammary tumor virus *pol*-related sequences in human DNA: characterization and sequence comparison with the complete murine mammary tumor virus *pol* gene. J. Virol. **57:**422–432.
3. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
4. **Guyader, M., M. Emerman, P. Sonigo, F. Clavel, L. Montagnier, and M. Alizon.** 1987. Genome organization and *trans*-activation of the human immunodeficiency virus type 2. Nature (London) **326:**662–669.
5. **Hartley, J. W., and W. P. Rowe.** 1975. Clonal cell lines from a feral mouse embryo which lack host-range restriction for murine leukemia viruses. Virology **65:**28–134.
6. **Hartley, J. W., and W. P. Rowe.** 1976. Naturally occurring murine leukemia viruses in wild mice: characterization of a new "amphotropic" class. J. Virol. **19:**19–25.
7. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26:**365–369.
8. **Johnson, M. S., M. A. McClure, D.-F. Feng, J. Gray, and R. F. Doolittle.** 1986. Computer analysis of retroviral *pol* genes: assignment of enzymatic functions to specific sequences and homologies with nonviral proteins. Proc. Natl. Acad. Sci. USA **83:**7648–7652.
9. **Kanaya, S., and R. J. Crouch.** 1983. DNA sequence of the gene coding for *Escherichia coli* ribonuclease H. J. Biol. Chem. **258:**1276–1281.
10. **Larder, B. A., D. J. M. Purifoy, K. L. Powell, and G. Darby.** 1987. Site-specific mutagenesis of AIDS virus reverse transcriptase. Nature (London) **327:**716–717.
11. **Levin, J. G., S. C. Hu, A. Rein, L. I. Messer, and B. J. Gerwin.** 1984. Murine leukemia virus mutant with a frameshift in the reverse transcriptase coding region: implications for *pol* gene structure. J. Virol. **51:**470–478.
12. **Merregaert, J., M. Janowski, and E. P. Reddy.** 1987. Nucleotide sequence of a radiation leukemia virus genome. Virology **158:** 88–102.
13. **Michel, F., and B. F. Lang.** 1983. Mitochondrial class II introns encode proteins related to the reverse transcriptases of retroviruses. Nature (London) **316:**641–643.
14. **Moelling, K., D. P. Bolognesi, H. Bauer, W. Busen, H. W. Plassmann, and P. Hausen.** 1971. Association of viral reverse transcriptase with an enzyme degrading RNA moiety of RNA: DNA hybrids. Nature (London) **234:**240–243.
15. **Omer, C. A., and A. J. Faras.** 1982. Mechanism of release of the avian retrovirus tDNA^{trp} primer molecule from viral DNA by ribonuclease H during reverse transcription. Cell **30:**797–805.
16. **Patarca, R., and W. A. Haseltine.** 1984. Sequence similarity among retroviruses—erratum. Nature (London) **309:**728.
17. **Pfeiffer, P., and T. Holm.** 1983. Involvement of reverse transcriptase in the replication of cauliflower mosaic virus: a detailed model and test of some aspects. Cell **33:**781–789.
18. **Power, M. D., P. A. Marx, M. L. Bryant, M. B. Gardner, P. J. Barr, and P. A. Luciw.** 1986. Nucleotide sequence of SRV-1, a type D simian acquired immune deficiency syndrome retrovirus. Science **231:**1567–1572.
19. **Rein, A., B. M. Benjers, B. I. Gerwin, R. H. Bassin, and D. R. Slocum.** 1979. Rescue and transmission of a replication-defective variant of Moloney murine leukemia virus. J. Virol. **29:** 494–500.
20. **Resnick, R., C. A. Omer, and A. J. Faras.** 1984. Involvement of retrovirus reverse transcriptase-associated RNase H in the initiation of strong-stop (+) DNA synthesis and the generation of the long terminal repeat. J. Virol. **51:**813–821.
21. **Roth, M. J., N. Tanese, and S. P. Goff.** 1985. Purification and characterization of murine retroviral reverse transcriptase expressed in *E. coli*. J. Biol. Chem. **260:**9326–9335.
22. **Rowe, W. P., W. E. Pugh, and J. W. Hartley.** 1970. Plaque assay techniques for murine leukemia viruses. Virology **42:**1136–1139.
23. **Sagata, N., T. Yasunaga, J. Tsuzuku-Kawamura, K. Ohishi, Y. Ogawa, and Y. Ikawa.** 1985. Complete nucleotide sequence of the genome of bovine leukemia virus: its evolutionary relationship to other retroviruses. Proc. Natl. Acad. Sci. USA **82:** 677–681.
24. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
25. **Schwartz, D. E., R. Tizard, and W. Gilbert.** 1983. Nucleotide sequence of Rous sarcoma virus. Cell **32:**853–869.
26. **Schwartzberg, P., J. Colicelli, and S. P. Goff.** 1984. Construction and analysis of deletion mutations in the *pol* gene of Moloney murine leukemia virus: a new viral function required for productive infection. Cell **37:**1043–1052.
27. **Seiki, M., S. Hattori, Y. Hirayama, and M. Yoshida.** 1983. Human adult T-cell leukemia virus: complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. Proc. Natl. Acad. Sci. USA **80:**3618–3622.
28. **Shinnick, T. M., R. A. Lerner, and J. G. Sutcliff.** 1981. Nucleotide sequence of Moloney murine leukemia virus. Nature (London) **293:**543–548.
29. **Sonigo, P., M. Alizon, K. Staskus, D. Klatzman, S. Cole, O. Danos, E. Retzel, P. Tiollais, A. Haase, and S. Wain-Hobson.** 1985. Nucleotide sequence of the Visna lentivirus: relationship to the AIDS virus. Cell **42:**369–382.

30. **Sonigo, P., C. Barker, E. Hunter, and S. Wain-Hobson.** 1986. Nucleotide sequence of Mason Pfizer monkey virus: an immunosuppressive D type retrovirus. Cell **45**:375–385.
31. **Summers, J., and W. S. Mason.** 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell **29**:403–415.
32. **Tanese, N., and S. P. Goff.** 1988. Domain structure of the Moloney murine leukemia virus reverse transcriptase: mutational analysis and separate expression of DNA polymerase and RNase H activities. Proc. Natl. Acad. Sci. USA **85**:1777–1781.
33. **Toh, H., H. Hayashida, and T. Miyata.** 1983. Sequence homology between retroviral reverse transcriptase and putative polymerases of hepatitis B virus and cauliflower mosaic virus. Nature (London) **305**:827–829.
34. **Toh, H., R. Kikuno, H. Hayashida, T. Miyata, W. Kugimiya, S. Inouye, S. Yuki, and K. Saigo.** 1985. Close structural resemblance between putative polymerase of *Drosophila* transposable genetic element 17.6 and *pol* gene product of Moloney murine leukemia virus. EMBO J. **4**:1267–1272.
35. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
36. **Verma, I. M.** 1975. Studies on reverse transcriptase of RNA tumor viruses. III. Properties of purified Moloney murine leukemia virus DNA polymerase and associated RNase H. J. Virol. **15**:843–854.
37. **Wain-Hobson, S., P. Sonigo, P. Danos, S. Cole, and M. Alizon.** 1985. Nucleotide sequence of the AIDS virus, LAV. Cell **40**:9–17.
38. **Zoller, M. J., and M. Smith.** 1984. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. DNA **3**:479–488.