Abortive Reverse Transcription by Mutants of Moloney Murine Leukemia Virus Deficient in the Reverse Transcriptase-Associated RNase H Function

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The reverse transcriptase enzymes of retroviruses are multifunctional proteins containing both DNA polymerase activity and a nuclease activity, termed RNase H, specific for RNA in RNA-DNA hybrid form. To determine the role of RNase H activity in retroviral replication, we constructed a series of mutant genomes of Moloney murine leukemia virus that encoded reverse transcriptase enzymes that were specifically altered to retain polymerase function but lack RNase H activity. The mutant genomes were all replication defective. Analysis of *in vitro* reverse transcription reactions carried out by mutant virions showed that minus-strand strong-stop DNA was formed but did not efficiently translocate to the 3' end of the genome; rather, the DNA was stably retained in RNA-DNA hybrid form. Plus-strand strong-stop DNA was not detected. These results suggest that RNase H normally promotes strong-stop translocation, perhaps by exposing single-stranded DNA sequences for base pairing. Four new DNA species were also detected among the reaction products. Analysis of these DNAs suggested that they were minus-strand DNAs formed from VL30 RNAs encoded by the mouse genome. We suggest that reverse transcriptase can initiate DNA synthesis at any one of four alternate tRNA primer-binding sites near the 5' ends of VL30 RNAs.

Reverse transcriptase (RT) is the enzyme in virions of retroviruses that is responsible for synthesis of a double-stranded DNA from the viral RNA genome (2, 42; for a recent review, see reference 11). All RTs are multifunctional proteins containing two major activities: a DNA polymerase activity capable of copying either RNA or DNA templates and an RNase activity, termed RNase H, capable of degrading RNA only in RNA-DNA hybrid form (26). The RT of Moloney murine leukemia virus (M-MuLV) is a simple monomeric enzyme containing the two activities in two distinct domains of the protein, with the DNA polymerase occupying the N-terminal two-thirds and RNase H occupying the C-terminal one-third of the molecule (40). Each domain can be expressed separately in *Escherichia coli* and shown to exhibit one or the other activity in the absence of its partner. The two activities are thought normally to act together *in vivo* to mediate all of the complex steps in the synthesis of a terminally redundant duplex DNA from the single-stranded RNA genome (10, 32).

Reverse transcription of the viral genome is initiated by elongation of a primer tRNA annealed to specific sequences in the viral RNA near the 5' end, the primer-binding site (pbs), and first forms a short DNA intermediate termed minus-strand strong-stop DNA (13, 43). This molecule must then translocate or jump to the 3' end of the genome, using a short repeated sequence in the viral RNA to promote base pairing, before elongation of the minus strand continues (6, 39). As the RNA genome enters RNA-DNA hybrid form, RNase H acts to degrade it; a polypurine tract near the 3' end of the genome remains resistant to degradation and serves as the primer for synthesis of the plus strand of DNA

(21, 31), yielding a second intermediate, termed plus-strand strong-stop DNA (25). This DNA must also translocate to the 3' end of its template before elongation can be completed. RNase H has been implicated in several of these steps, including removal of the tRNA primer (28) and formation and removal of the plus-strand RNA primer (5, 21, 29-31, 36). It is unclear, however, whether RNase H is needed at other times. For example, it is unclear whether RNase H must remove the genomic RNA hybridized to minus-strand strong-stop DNA to permit it to translocate to the 3' end of the genome or whether strong-stop DNA can transiently denature from the 5' end of the genome and renature with the 3' end of the genome to permit its elongation. Biochemical studies suggest that the 5' part of the genomic RNA is degraded during reverse transcription (8, 9), and it is plausible that this degradation is essential.

To determine the effects of elimination of RNase H activity during reverse transcription, we generated a series of mutants of M-MuLV with specific mutations in the RT domain of the viral *pol* gene. These mutations were previously shown to abolish RNase H activity and have little or no effect on the DNA polymerase function of M-MuLV RT expressed as a fusion protein in *E. coli* (40). Analysis of the reverse transcription products made by virion particles derived from the mutant genomes showed that minus-strand strong-stop DNA was synthesized normally but that this species was retained stably in RNA-DNA hybrid form and did not translocate efficiently to permit further elongation. The results suggest that RNase H is indeed needed for minus-strand strong-stop translocation. In addition, several new DNA species were detected. The structures of these DNAs suggest that they are encoded by viruslike RNA genomes termed VL30 RNAs (3, 15) and that RT has the capability of initiating synthesis at any one of four alternate pbs near the 5' ends of these RNAs.

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MATERIALS AND METHODS

Cells and DNA-mediated transformations. NIH 3T3, Clone 4, Rat-2, and XC cells were as described previously (35) and were maintained in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% newborn calf serum (MA Bioproducts). Viral DNAs were tested for replication competence by transformation of NIH 3T3 cells by the DEAE-dextran method (24); production of infectious virus was assessed by RT assays of the supernatant medium (12) and by XC plaque assay (33). Stable virus-producing cell populations were derived after calcium phosphate-mediated cotransformation (44) with a mixture of proviral DNA and pSV2neo DNA (38) by growth in G418 (GIBCO; 400 µg/ml). Clonal cell lines were obtained from such populations by single-cell cloning in 96-well trays and screening for release of high levels of virion-associated RT (12). Infections with virus were carried out for 2 h in the presence of 8 µg of Polybrene per ml. Low-molecular-weight DNA was extracted 24 h postinfection (14), and viral DNA sequences were detected by agarose gel electrophoresis and Southern blot analysis (37).

DNA manipulations. Cloning operations were carried out by standard protocols (34). Mutant plasmids H7, H2, H12, MC7, H27, M32, M42, H8, and M4 contain *EcoRI* linker insertions in parental expression plasmid pSH1 and were described previously (40). Plasmid pT11 contains a full-length proviral copy of the M-MuLV genome with flanking DNA (20), and pNCA contains the same provirus without flanking DNA in a portion of the pBR322 vector (7). To transfer the mutations from the pSH1 derivatives to a complete provirus, a 1.1-kb fragment was excised from each mutant plasmid by cleavage with *SalI* plus *HindIII*, purified, and used to replace the corresponding wild-type fragment in pT11.

Endogenous reverse transcription reactions in virion particles. Supernatant culture fluid was collected from producer cell lines and clarified, and the virions were pelleted by centrifugation through a 25% sucrose cushion as described previously (41). The virions from 40 ml of culture fluid were suspended in 200 µl of TNEP buffer (100 mM Tris-Cl [pH 8.3], 100 mM NaCl, 1 mM EDTA, 0.01% Nonidet P-40) and stored at -70°C. Endogenous reactions to detect minus-strand strong-stop DNA were carried out by using 20 µl of virus preparations in 50 µl of buffer (50 mM Tris-Cl [pH 8.3], 50 mM NaCl, 6 mM MgCl₂, 0.01% Nonidet P-40; 1 mM dithiothreitol; dCTP, dATP, and dGTP, each at 1 mM; 2.5 µM [³²P]dTTP at 400 Ci/mmol) and incubated for 30 min at 37°C. The reactions were terminated by addition of 10 µl of 10% sodium dodecyl sulfate (SDS) and 2 µl of 0.5 M EDTA, and the reaction products were extracted twice with phenol and collected by ethanol precipitation. Endogenous reactions to detect plus-strand strong-stop DNA were carried out by using 10 µl of wild-type virus or 40 µl of mutant virus preparations in 70-µl reactions containing the buffer described above but with dCTP, dATP, and dGTP, each at 2 mM, and 1 mM [³²P]dTTP at 1 Ci/mmol and incubated for 2 h at 40 to 41°C. In some reactions, actinomycin D was included at 100 µg/ml (final concentration). Reactions were terminated by addition of SDS and EDTA as described above, 0.5 µg of carrier RNA was added, and proteins were digested with 4 µg of proteinase K for 30 min at 37°C. The nucleic acids were phenol extracted and collected by two ethanol precipitations. In some cases, the reaction products were incubated in 0.33 N NaOH for 20 min at 55°C before the

final ethanol precipitation to remove RNA primers. The reaction products were suspended in TE (10 mM Tris-Cl [pH 7.5], 1 mM EDTA), electrophoresis sample buffer was added, and the samples were heated at 90°C for 10 min before analysis by electrophoresis on 5, 7.5, or 10% polyacrylamide gels. After electrophoresis, the gels were dried and exposed by autoradiography.

DNA size standards for electrophoresis included restriction enzyme digestion products of various plasmid DNAs. In some experiments, an end-labelled DNA fragment was produced by cleavage of pNCA with *NdeI*, treatment with phosphatase and T4 polynucleotide kinase in the presence of [³²P]ATP, and recleavage with *ClaI*. This DNA was then redigested with various enzymes and denatured to form marker DNAs of the same sequence as authentic minus-strand strong-stop DNA and its elongated products.

Enzyme digestions of labelled DNA species. After synthesis in endogenous DNA reactions, some DNA samples were treated with RNase A (2 µg in 50 µl) in low-salt buffer (TE) or high-salt buffer (TE containing 0.5 M NaCl) for 30 min at 37°C. The enzyme was inactivated by treatment with diethylpyrocarbonate (1 µl) at 37°C for 10 min. The samples were either diluted into electrophoresis sample buffer and disaggregated without denaturation by mild heating (50°C for 5 min) or diluted into buffer containing formamide and fully denatured (90°C for 10 min).

DNAs were recovered from dried gels by rehydrating the gel slices in elution buffer (23), crushing the gel, and filtering the supernatant through glass wool with centrifugation. The DNAs were precipitated with ethanol and suspended again in TE. To recover DNAs from wet gels, the DNA was passively eluted by soaking gel slices in TE for 48 h, precipitating DNA in the eluate with ethanol, and suspending it in TE. In some experiments, the DNAs were subjected to further digestions, diluted into electrophoresis sample buffer, and analyzed by electrophoresis. For digestions of single-stranded DNAs by *EcoRI*, the DNAs were annealed to an *EcoRI* linker (sequence, 5'-GGAATTCC-3') by incubation at 37°C for 20 min in 150 mM NaCl-10 mM MgCl₂-10 mM Tris-Cl (pH 7.5) and then digested with *EcoRI* at 25°C for 6 h in the same buffer.

Metabolic labelling of viral proteins and immunoprecipitation. Virion proteins were labelled by incubating cells (2 × 10⁶/10-cm-diameter dish) in 5 ml of methionine-free medium with dialyzed calf serum containing 150 µCi of [³⁵S]methionine for 12 h. The supernatant culture fluid was collected, clarified, and filtered (Gelman Acrodisc; 0.45-µm pore size), and virions were collected by centrifugation (25,000 rpm for 4 h in an SW28s rotor) through a cushion of 25% sucrose (5 ml). The virions were suspended and disrupted in 40 µl of sample buffer (60 mM Tris-Cl [pH 6.8], 6 mM EDTA, 100 mM β-mercaptoethanol, 1% SDS, 10% glycerol) and diluted 10-fold with immunoprecipitation buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM sodium phosphate [pH 7.5], 100 mM NaCl). The lysates were clarified, and the viral proteins were incubated with specific antisera and collected by immunoprecipitation with Pan-sorbin. The antisera used included anti-pSC1, a rabbit polyclonal serum directed against M-MuLV *pol* gene products (41); and NCI 77S-158 serum, a polyclonal goat serum reactive against M-MuLV p30 (CA) protein. The proteins were analyzed by electrophoresis on 10% polyacrylamide gradient gels and visualized by fluorography.

FIG. 1. Positions of mutations in the *pol* gene of M-MuLV. The top diagram shows the complete M-MuLV provirus, with map coordinates indicated in kilobases (kb). The *pol* gene is shown divided into three domains that encode the PR, RT, and IN functions. The RT domain is shown in expanded view below, subdivided into the regions that encode the DNA polymerase and RNase H activities. The positions of the linker insertion mutations are indicated by the open triangles; the positions and extents of the deletion mutations are indicated by the shaded bars. The position of the 5' edge of each mutation and the net number of base pairs inserted or deleted are indicated below. fs, frameshift.

RESULTS

Construction of mutant viral genomes and test for replication. We have previously described the analysis of a panel of mutants of the M-MuLV RT gene expressed in bacteria as a large TrpE-RT fusion protein (40). Each mutant enzyme, containing an *EcoRI* linker insertion, was tested for DNA polymerase and RNase H activity. Mutations in the 5' two-thirds of the gene generally eliminated polymerase activity without affecting RNase H, while those in the 3' one-third of the gene generally eliminated RNase H without abolishing polymerase activity. We selected a total of nine of these mutants for this study, including insertion and deletion mutants (Fig. 1). One mutant, H2, with an in-frame insertion near the boundary between the domains, retained both activities, while the rest lacked detectable RNase H activity when assayed *in vitro*. In five cases, the insertions created a frameshift mutation, blocking expression of the integrase (IN) function lying downstream. The exact number of base pairs lost in deletion mutant H12 is not known. DNA fragments containing each of the selected insertion mutations were excised from the expression constructs and used to replace the corresponding fragment of a wild-type infectious copy of the M-MuLV proviral DNA.

The biological activity of the mutants was tested by introducing the cloned DNAs into NIH 3T3 cells in culture by using DEAE-dextran (24). In this procedure, the viral DNAs are expressed only transiently and detection of replicating virus requires multiple rounds of viral growth. All of the mutant DNAs were defective for replication, judged by XC plaque assay and assays for released RT activity. Thus,

FIG. 2. Representative RT assays of culture medium supernatants harvested from stable producer cell lines expressing M-MuLV proviral DNAs. Reaction products were spotted onto DEAE paper, washed, and exposed to X-ray film. Dots: 1, no medium; 2, medium from NIH 3T3 cells carrying SV2neo DNA alone; 3 to 6, medium from cells cotransformed with SV2neo DNA plus the indicated mutant DNA; 7, medium from cells transformed with wild-type M-MuLV DNA clone T11; 8, medium from clone 4 cells infected with wild-type M-MuLV.

even the H2 mutant, which retained both enzymatic activities when assayed *in vitro*, was replication defective *in vivo*. This suggests that the viral *pol* gene is more sensitive to mutations for replication *in vivo* than for simple enzymatic functions on synthetic templates measured *in vitro*. In addition, the results suggest that the RNase H domain is essential for some aspect of replication.

Establishment of cell lines releasing mutant virions. To characterize the mutant viruses in greater detail, we established cloned cell lines that stably express the viral gene products. NIH 3T3 cells were cotransformed by a mixture of pSV2neo and each of five mutant DNAs (H7, H2, H27, M32, and M4), and recipient cells were isolated after selection with medium containing G418. Pooled cell populations obtained with each mutant released substantial levels of RT, showing that the mutant genomes all directed the expression of at least the *gag* and *pol* gene products. Single-cell clones were prepared from populations carrying four of the mutants and screened for RT activity (12). In this way, cloned cell lines releasing high levels of each of the four mutant virions were obtained (Fig. 2). There was considerable variation in the level of virion production in the various clones; some of the variation is probably inherent to the mutants, while some is attributable to the variable expression of proviruses introduced by DNA-mediated transformation.

The viral proteins present in the virions produced by each mutant were examined. Cell lines were metabolically labelled with [³⁵S]methionine, and the virions were harvested from the supernatant medium, purified, and disrupted with detergent. Portions of the viral proteins were immunoprecipitated with antiserum specific for the *gag* CA-encoding domain (NCI 77S-158) or the *pol* RT and IN domains (anti-SC1 serum [41]), and the samples were then analyzed by SDS-polyacrylamide gel electrophoresis, followed by autoradiography (Fig. 3). All four mutant virions contained fully processed *gag* products, evidenced by the presence of mature CA protein and only very low levels of the uncleaved Pr65^{gag} precursor, similar to the wild-type virus. In each case, *pol* polypeptides with molecular weights consistent with the structure of the mutation were detected. Mutant M4, containing an insertion of 15 bp that preserves the reading frame, synthesized both RT and IN proteins of sizes indistinguishable from those of the wild type. For mutant H7, the RT protein was 53 kDa; for H27, the RT was 62 kDa; and for M32, it was 58 kDa. Virions of mutants H7, H27, and M32 contained no detectable IN protein, as predicted by the presence of a frameshift mutation in their RT domains. These results suggest that the RT-related proteins encoded

FIG. 3. SDS-polyacrylamide gel electrophoresis of [35 S]methionine-labelled virion proteins. NIH 3T3 cells expressing the indicated DNAs were metabolically labelled with [35 S]methionine, and virions were harvested from the supernatant culture medium. Virions were disrupted, aliquots of the samples were immunoprecipitated, and the proteins were analyzed by electrophoresis and fluorography. Lanes: V, total virion proteins without immunoprecipitation; G, proteins immunoprecipitated with anti-*gag* serum; P, proteins immunoprecipitated with anti-*pol* serum. The positions of migration of molecular size standards are indicated at the left in kilodaltons. The positions of *gag* protein CA, *pol* protein IN, and the various RT proteins are indicated on the right.

by all four of the mutants were successfully expressed, incorporated into virion particles, and processed by the viral protease at the processing sites available in the mutant precursor proteins.

Reverse transcription by mutant virions in vivo and in vitro.

To define the nature of the defect in the mutant viruses, virions were collected from the four mutant producer cell lines and used for acute infection of NIH 3T3 and Rat-2 cells. Low-molecular-weight DNAs were harvested from the infected cells at 24 h postinfection and tested for viral sequences by Southern blot analysis. Whereas high levels of all three viral DNA forms that are normally synthesized were found in cells infected with wild-type virus, none of these species were detected in either cell type infected with the four mutants (Fig. 4). Thus, all four mutant viruses were unable to generate full-length reverse transcripts in vivo.

The complete process of reverse transcription can be carried out in vitro by M-MuLV virion particles incubated with detergent and the four deoxyribonucleotides, and various DNA intermediates can be detected (32). Reactions carried out for short times in low nucleoside triphosphate concentrations yield predominantly minus-strand strong-stop DNA, while prolonged incubation in high nucleoside triphosphate concentrations permits its translocation, formation of long minus-strand DNAs, and synthesis of plus-strand strong-stop DNA. To determine whether the mutant virions could carry out any of these steps, we harvested virions from cell lines producing the four mutants and wild-type virus and carried out reverse transcription reactions under both conditions by using 32 P-labelled nucleoside triphosphate to label the newly synthesized DNA.

Virions were first incubated with low nucleoside triphosphate concentrations to test for formation of minus-strand strong-stop DNA. The newly synthesized DNA was isolated, treated with RNase A in low salt concentrations, heated to denature any duplex structures, and examined by

FIG. 4. Southern blot analysis of unintegrated viral DNA formed in recently infected cells. Virus supernatants were harvested from cell lines stably expressing various M-MuLV mutants and used to infect either NIH 3T3 (lanes 1 to 6) or Rat-2 (lanes 7 to 12) cells. Low-molecular-weight DNA was isolated after 24 h and analyzed by agarose gel electrophoresis and Southern blotting, hybridizing with a virus-specific probe. The positions of migration of the 8.8-kb linear genomic DNA and the two circular DNAs are indicated at the right. DNAs detected near the top of the gel were contaminating cellular sequences homologous to the probe.

gel electrophoresis and autoradiography (Fig. 5). All four mutant virions lacking the RNase H function made high levels of the minus-strand strong-stop DNA. Low levels of shorter DNAs, originally termed weak-stop DNAs and arising from pausing by RT, were visible in both mutant and wild-type reaction products. Normalizing the yield of minus-strand strong-stop DNA to the level of input virions, as judged from RT assays on exogenous templates, suggests that nearly normal levels of the DNA were formed. Thus, genomic RNA, although not directly measured, must be present at nearly normal levels in the particles. This result demonstrates that the RNase H function is not required for incorporation of viral RNA into the virion particle, placement of the primer tRNA on the viral RNA, or elongation from the primer to initiate DNA synthesis.

Incubation of virions in high nucleoside triphosphate concentrations for longer times revealed significant differences in the products formed by the wild-type and mutant virions (Fig. 6). In the wild-type virions, the level of minus-strand strong-stop DNA decreased and large amounts of long DNAs were formed, indicating that the DNA had translocated and elongated (Fig. 6, lanes 5 and 6). In the mutants, the bulk of the strong-stop DNA persisted stably and only small amounts of longer material were formed. This suggests that the minus-strand strong-stop DNAs synthesized by the mutants were only poorly able to translocate to permit further synthesis.

Four new discrete DNAs, longer than the minus-strand strong-stop DNA, were present in the products of the four mutants (Fig. 6). The sizes of the DNAs made by all of the

FIG. 5. DNAs synthesized by virion particles during in vitro reverse transcription reactions at low nucleoside triphosphate concentrations. Virions were collected from cell lines expressing the indicated virus mutants and incubated in reverse transcription reaction cocktail. The labelled DNA products were recovered, treated with RNase A, and analyzed by polyacrylamide gel electrophoresis, followed by autoradiography. The position of migration of the minus-strand strong-stop DNA is indicated.

mutants were indistinguishable; comparison with marker DNA fragments suggested sizes of approximately 220, 235, 270, and 305 nucleotides (nt). The absolute levels of the four species and their relative abundances were different among the four mutants and were reproducible and characteristic of each mutant. Thus, mutant H7 always generated the highest level of the four DNAs and mutant M4 generated the lowest level. Mutant H27 formed predominantly the two smallest species, while mutant M32 generated high levels of the 235-nt DNA. The structures of these DNAs are considered below.

Analysis of minus-strand strong-stop DNAs synthesized in mutant virions. To help determine why the minus-strand strong-stop DNAs of the mutants were poorly translocated and chased into longer DNAs, the properties of the DNAs were assessed by nuclease digestion and electrophoresis. Reactions were run in low nucleoside triphosphate conditions as before, and the products were then exposed to RNase A in either a low or a high salt concentration.

FIG. 6. DNAs synthesized by virion particles in vitro after prolonged incubation at high nucleoside triphosphate concentrations. Virion preparations and gel analysis were as in Fig. 5. The positions of migration of single-stranded DNA markers are indicated at the right. The minus-strand strong-stop DNAs and the four novel species a to d are indicated at the left.

Digestion in a low salt concentration would degrade any RNA linked or annealed to the DNA, while digestion in a high salt concentration would leave intact any RNA that was present in duplex or hybrid form. After digestion, the samples were divided into two aliquots; one was heated in buffer containing formamide to denature duplex structures as much as possible, and the other was left undenatured. The DNAs were analyzed by electrophoresis on nondenaturing polyacrylamide gels and autoradiography.

Analysis of the samples after heat denaturation showed that the mutant minus-strand strong-stop DNAs were similar in size and structure to the wild type. Digestion with RNase in a low salt concentration generated the expected DNAs of 145 nt, free of the tRNA primer, for both the mutant and the wild type (Fig. 7, lanes 12, 14, 16, 18, and 20). Digestion in a high salt concentration generated a species that migrated at approximately 163 nt, or 18 nt longer, as expected for protection of 18 nt of the primer tRNA at the 5' end of the DNA; these bases of the tRNA are paired with the pbs of the genomic RNA in an RNA-RNA duplex (lanes 11, 13, 15, 17, and 19). Thus, the minus-strand strong-stop DNAs of the mutant and wild-type viruses are of similar size, have the primer tRNA attached, and have the pbs portion of the tRNA base paired.

FIG. 7. Analysis of minus-strand strong-stop DNAs synthesized by wild-type and mutant virions. Reverse transcription reactions were performed, and the DNA products were isolated and treated with RNase A in either a high (H; odd-numbered lanes) or a low (L; even-numbered lanes) salt concentration. The samples were then analyzed without denaturation (lanes 1 to 10) or with heat denaturation (lanes 11 to 20) by polyacrylamide gel electrophoresis. The positions of the 163- and 145-nt single-stranded species are indicated at the right. The positions of the slow (lane 1) and fast (lanes 3, 5, 7, and 9) forms are indicated at the left.

Analysis of the samples without heat denaturation showed that the mutant DNAs, although similar in size, were present in a hybrid form different from that of the wild-type DNA. Treatment of the wild-type products with RNase A in a high salt concentration yielded a band that migrated even more slowly than the single-stranded DNA that retained 18 nt of covalently attached tRNA (Fig. 7, lane 1). We suggest that this species was the 145-nt strong-stop DNA with the 18-nt stretch of the tRNA attached and with fragments of genomic RNA—notably including 18 nt of the pbs—still annealed to it. It is not unexpected that such partially single- and double-stranded species would migrate aberrantly and slowly. Upon heating, this species converted to the 163-nt form (Fig. 7, lane 11). Thus, the wild-type strong-stop DNA was probably largely in single-stranded form but retained the tRNA at the 5' end, and 18 nt of this tRNA was still annealed to genomic RNA and protected from digestion. In contrast, treatment of the reaction products of three of the mutants (H7, H27, and M32) with RNase A in a high salt concentration yielded almost exclusively a single novel band which migrated more rapidly than the single-stranded 145-nt DNA (Fig. 7, lanes 3, 5, and 7). We suggest that this species is DNA in RNA-DNA duplex form (see below). Mutant M4 yielded a mixture of two bands, one in the position of the wild-type product and one in the position seen for the other mutants (Fig. 7, lane 9). Treatment of all of the reaction products with RNase A in a low salt concentration removed all of the RNA, converting them to 145-nt species that comigrated with the denatured DNAs described above (Fig. 7, lanes 2, 4, 6, 8, and 10).

A summary of the proposed structures and the treatments that were used to form them is given in Fig. 8A. The various species detected are numbered with the boxed labels 1 to 8. To confirm the assignments of these various species, DNAs in the bands from wild-type and mutant H7 virions were eluted from gels, recovered, and subjected to further digestions and gel analysis. Each sample was divided into two aliquots; one aliquot was treated with RNase A in a low salt

FIG. 8. Analysis of the minus-strand strong-stop DNA species synthesized by wild-type and mutant virions. (A) Flow chart for generation of the DNA species detected in the experiment described in Fig. 7 and proposed structures. DNAs are indicated by heavy lines, and RNAs are indicated by thin lines; duplex regions are hatched. Arrowheads indicate the 3' ends of the strands. The primer tRNA is indicated by the folded structure. Rows: top, proposed structures as formed in virions; middle, proposed structures after treatment with RNase A in a low (left) or a high (right) salt concentration; bottom, positions of migration of DNAs with or without heat denaturation (Δ). The various species are labelled 1 to 8 by the boxed numbers. (B) Analysis of DNA species synthesized by wild-type virus and mutant H7 by further treatments. Some of the DNA species indicated in panel A were isolated from the gel in Fig. 7 and subjected to further analysis. The DNAs were incubated with or without RNase as indicated, heat denatured, and analyzed by electrophoresis in polyacrylamide. The positions of migration of the 163- and 145-nt species, judged from marker DNAs, are indicated at the left.

concentration, and the other was untreated. Both aliquots were then heat denatured and analyzed by gel electrophoresis as before (Fig. 8B). The 145-nt species recovered from the original digestions performed in a low salt concentration (species 1, 2, 5, and 6 in Fig. 8A) were unaffected by the second RNase A digestions, continuing to migrate as 145-nt DNA. Thus, as expected, these species were DNA free of added RNA. The 163-nt species recovered from the original digestions performed in a high salt concentration (species 3 and 7 in Fig. 8A) were affected: without digestion they ran at 163 nt, and after secondary digestion in a low salt concentration they shifted to 145 nt. The small amount of 145-nt DNA seen before digestion presumably reflects RNA loss during handling. Thus, as expected, these DNAs contained 18 nt of the tRNA primer in duplex form. Finally and most importantly, the fast-migrating form recovered from the mutants (species 8 in Fig. 8A) was affected by redigestion: upon being heated alone, the DNA ran as a 163-nt species; upon digestion and heating, it ran as a 145-nt species. Thus, the properties of the fast-migrating form were consistent with those expected for the 163-nt form of strong-stop DNA

FIG. 9. Reverse transcription products synthesized by wild-type and mutant virions in the presence of actinomycin D. Virions were incubated in reactions as described in the legend to Fig. 6, in the presence of high nucleoside triphosphate concentrations and with or without actinomycin D (Act D), as indicated at the top. The wild-type reactions were performed with levels of virions comparable to those of the mutants or at five times these levels. The positions of migration of the single-stranded marker DNAs in lane M are indicated at the right. The normal minus-strand strong-stop DNA and the four novel species are indicated at the left. The synthesis of all five DNA species was resistant to actinomycin D.

annealed to RNA. These overall results support the notion that the mutant strong-stop DNAs are retained in RNA-DNA hybrid form.

Analysis of novel DNA species formed by mutant virions. The analysis described above suggested that in reverse transcription reactions carried out in low nucleoside triphosphate concentrations, mutant virions deficient in RNase H generated only minus-strand strong-stop DNA and retained this intermediate in duplex with RNA. When reactions were run in high nucleoside triphosphate concentrations, minus-strand strong-stop DNA persisted but four novel, longer species were seen (Fig. 6). To determine the origin and structure of these four species, further analyses were performed. The polarity of the DNAs—i.e., whether they were minus- or plus-strand DNAs—was addressed by carrying out reactions in the presence or absence of actinomycin D. This drug, which inhibits DNA-dependent but not RNA-dependent DNA synthesis, has no effect on initial minus-strand DNA synthesis but blocks formation of all plus-strand DNA species (32). Analysis of the products of the four mutants showed that addition of actinomycin D had no effect on the appearance of either minus-strand strong-stop DNA or the four longer DNAs (Fig. 9). The relative abundance of the

FIG. 10. Analysis of DNAs synthesized by mutant virions for covalently attached RNA sequences. Virions were collected from cell lines transformed by the indicated mutant DNAs, reverse transcription reactions were carried out as described in the legend to Fig. 6, and the reaction products were isolated. The DNAs were incubated with or without RNase A in a low salt concentration as indicated, denatured with heat, and analyzed by polyacrylamide gel electrophoresis. The positions of migration of single-stranded marker DNAs are indicated at the right. The products seen without RNase (species A, B, C, and D and the 210-nt minus-strand strong-stop DNA) and with RNase (species a, b, c, and d and the 145-nt minus-strand strong-stop DNA) are indicated at the left.

species for each mutant was as seen previously (Fig. 6). This result strongly suggests that the four DNAs were all minus-strand DNAs.

The minus-strand strong-stop DNA of wild-type M-MuLV contains 65 ribonucleotides, the host proline tRNA, attached to its 5' end; treatment with alkali or RNase in a low salt concentration before electrophoresis thus shifts its mobility from that of a species of about 210 nt to one of 145 nt. To determine whether the four novel DNAs were also primed by RNA, reactions were run in high nucleoside triphosphate concentrations as before and the samples were divided into two aliquots. One was left untreated, and the other was treated with RNase A in a low salt concentration. The samples were then heat denatured and examined by electrophoresis as before. As expected, the minus-strand strong-stop DNAs shifted to smaller forms by approximately 70 nt upon RNase A treatment (Fig. 10). In addition, each of the four new DNAs shifted to correspondingly smaller forms; the characteristically different levels of the four DNAs

FIG. 11. Restriction enzyme analysis of DNAs synthesized by mutant H7 virions. Reaction products were subjected to alkali treatment to remove RNA primers, digested, and analyzed by polyacrylamide gel electrophoresis. (A) Digests of separated species a to d and minus-strand strong-stop DNA with *SacI*, showing production of common bands from a to d (arrows) and a common partial digestion product (broken arrow). (B) Unfractionated reaction products were annealed to *EcoRI* linkers and digested with *EcoRI*. The electrophoresis showed cleavage of species a and b to form a common band (a',b') and a 35-nt product (arrow). The numbers to the right of panel A and the left of panel B indicate the positions of the molecular size markers.

allowed assignment of the shifted bands, showing that all four shifted down by similar amounts. Measurement of the sizes of the DNAs, judged from single-stranded marker DNAs, showed that all four were shifted by approximately 65 to 75 nt. These results suggest that the four DNAs were probably all primed by a tRNA at their 5' termini.

To test whether the four DNAs were derived from the M-MuLV genome, the labelled DNAs were annealed to cloned fragments of M-MuLV DNA and tested for protection from digestion by S1 nuclease. Single-stranded DNA from four separate M13 clones containing M-MuLV sequences which together span the entire genome were individually hybridized to the unfractionated labelled DNAs and then subjected to S1 digestion by using standard procedures (34). None of the four portions of the M-MuLV genome was able to protect the four novel species from digestion (data not shown). The minus-strand strong-stop DNA present in the reaction products was fully protected by fragments of the M-MuLV genome containing the long terminal repeat. These results suggested that the four DNAs were encoded by another virus or viruslike element.

To help determine the origin of these DNAs, they were subjected to restriction enzyme analysis. The reaction products formed by mutant H7 virions and wild-type virions were treated with alkali to remove RNA, heat denatured, and either isolated from preparative gels before digestion or digested directly. In some cases, the DNA was annealed to single-stranded oligonucleotides or fragments of M-MuLV DNA to form double-stranded DNAs suitable for digestion by restriction enzymes. After digestion, the DNAs were denatured and analyzed by gel electrophoresis and autoradiography as before.

The undigested DNAs of mutant H7 migrated in the expected positions seen previously (Fig. 11A and B). Treatment of fragments a, b, c, and d with *SacI* generated new fragments shorter by about 90 nt than the parent DNA in each case, as well as three common products (Fig. 11A, lanes 2 to 5, arrows). The sizes of the common bands (approximately 40, 45, and 85 nt) and their relative intensities suggest that all four of the parent species had two *SacI* sites in common; the 40- and 45-nt fragments would be complete digestion products, and the 85-nt fragment would

FIG. 12. Summary of proposed structures for DNA species formed in RNase H-deficient virions. DNAs are indicated by heavy lines, and RNAs are indicated by light lines. DNAs are shown aligned with the genomic RNA template for their synthesis; the R, U5, and U3 segments of the genome are indicated. The major VL30 products (A to D) are minus-strand strong-stop DNAs with different 5' ends and common 3' ends. The single M-MuLV product is the classical minus-strand strong-stop DNA. A_n, poly(A) sequence.

be a partial digestion product. As expected from the S1 results described above, hybridization of the starting DNAs to cloned M-MuLV DNA did not improve the efficiency of cleavage (data not shown). Examination of the sequences of murine retroviral DNAs and related elements for the two *SacI* sites revealed a candidate origin for these species: VL30, or viruslike 30S, elements. These elements, endogenous to the mouse genome, encode abundant 30S RNAs that are efficiently packaged into retroviral virions and reverse transcribed (3, 15). Although not all VL30 genomes are identical (16–19), in many murine VL30 genomes the R region of the long terminal repeat contains a conserved pair of *SacI* sites separated by 41 nt (1, 27); in particular, cleavage of VL30 strong-stop DNAs would be predicted to yield precisely the bands observed (Fig. 12). Both *SacI* sites in these VL30 DNAs are contained in identical 10-bp palindromes, suggesting that cleavage of the single-stranded species is promoted by intramolecular pairing between the two sites.

To confirm that the four species were derived from VL30 genomes, an additional digestion was performed. The U5 regions of VL30 DNAs often contain two *EcoRI* sites, separated by 35 nt, located such that larger strong-stop DNAs would be cleaved (1, 27). Treatment of the unfractionated reaction products with *EcoRI* showed that the larger two DNAs, species a and b, were indeed digested to yield one smaller fragment (Fig. 11B, lane 3, a', b') and a 35-nt fragment (arrow); the smaller species, c and d, were unaffected. All of the data obtained are consistent with the

model presented in Fig. 12. The four larger DNA species are related VL30 minus-strand species primed by small RNAs the size of a tRNA. The mapping results suggest that they have different 5' ends and share a 3' end that maps at the 5' end of the VL30 RNA. Examination of the nucleotide sequences in this portion of some VL30s (NVL-3-type clones) (1, 4, 27) revealed the presence of four duplicated copies of the right edge of U5, three of which are followed by a potential tRNA^{Gly} pbs. This observation suggests that four distinct minus-strand strong-stop DNAs can be formed on this RNA and that these species accumulate in the mutants along with M-MuLV strong-stop DNA.

DISCUSSION

On the basis of biochemical analysis of retroviral reverse transcription and the activities of the RNase H enzyme, it has been surmised that the enzyme probably carries out several essential steps in replication. The genetic results described here confirm that the RNase H activity of retroviral RT, as expected, is an essential viral function. The analysis of mutant virions also shows that several steps do not require this activity: the RNase H domain of RT is not required for binding of the primer tRNA to the template, initiation of DNA synthesis, or formation of minus-strand strong-stop DNA. The mutant virions, however, did not efficiently chase the minus-strand strong-stop DNA into longer forms, even upon prolonged incubation (Fig. 6, 9, and 10). The analysis thus suggests that the first role that the enzyme normally plays is to promote translocation of minus-strand strong-stop DNA.

When RNase treated in a low salt concentration, heat denatured, and analyzed by gel electrophoresis, the minus-strand strong-stop DNA made by the mutants migrated normally (at 145 nt), behaving like the wild type. When RNase treated in a high salt concentration and heat denatured, both mutant and wild-type molecules also ran identically (at 163 nt), suggesting that in both of them the primer tRNAs were retained and paired with the 18-nt pbs. When treated with RNase A in a high salt concentration and examined without denaturation, however, the mutant DNAs ran as a fast-migrating form very different from the wild type. This form converted to the 163-nt form on heating and to the 145-nt form on RNase treatment in a low salt concentration, implying that it consisted of DNA paired to RNA. The results suggest that the minus-strand strong-stop DNA of the mutants might be retained in duplex form with the RNA genome. The failure of the strong-stop DNAs to translocate efficiently may be due to this retention as a hybrid. This idea is supported by recent reconstructions of template switch reactions, in which RNase H-deficient RTs showed significant reductions in switching efficiency (22).

Upon prolonged incubation, the mutant virions formed four discrete novel DNAs. These DNAs were resistant to actinomycin D present during synthesis and thus were probably of minus-strand polarity and were primed by RNAs similar in size to tRNAs. The analysis of these DNAs suggested that they were derived from the mouse VL30 RNAs, a family of abundant RNAs that are efficiently copackaged into virion particles (3, 15). VL30 genomes are of diverse sequences, and there may be more than 100 copies of these sequences in the mouse genome; it is thus intriguing that only four discrete species, all clearly related by their common restriction maps, are formed in the virions. While they could be derived from four distinct VL30 genomes, three of them, and possibly all four, may be derived from a

single VL30 RNA very similar in sequence to the NVL-3-type clones (1, 4, 27). This VL30 genome contains three tandem potential pbs for tRNA^{Gly}; the positions of these sites account perfectly for the formation of species a, b, and c. Each of these pbs stretches is next to a proper inverted repeat sequence for viral integration. Thus, all could be alternatively used to form proviral DNAs with long terminal repeats of different sizes. A fourth site, less well conserved, present 15 nt closer to the 5' end of the genome than the primer for species c, could account for the formation of species d; however, a different RNA containing the VL30 *SacI* sites may also be the source of this DNA. It is interesting that each of the mutant RTs generated different relative amounts of the four species. We suppose that the mutant enzymes utilize the four possible pbs sequences at different efficiencies, perhaps interacting differently with the template during annealing of the tRNA. Careful examination of the DNA products formed by wild-type virions has revealed the presence of low levels of the same species, suggesting that these VL30 strong-stop DNAs are formed in normal infections; we presume that these DNAs are hard to detect because they are normally translocated and elongated.

Perhaps the most significant finding is the fact that all of the DNAs formed by the RNase H mutants—both the minus-strand strong-stop DNA and the four novel species—are not efficiently translocated and elongated. It thus seems very likely that RNase H is required to promote translocation and that degradation of the genomic RNA from the 5' end of the genome is the critical step. Clearly, other steps in reverse transcription—such as formation of plus-strand primers—would also be blocked by the lack of this activity, but translocation is temporally the first step blocked. It will be important to determine the level of RNase H activity required for efficient translocation and whether specificity for structure or sequence, or other activities, is required for translocation.

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