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Defects in Moloney Murine Leukemia Virus Replication Caused by a Reverse Transcriptase Mutation Modeled on the Structure of *Escherichia coli* RNase H

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We have studied a mutant Moloney murine leukemia virus with a deletion in reverse transcriptase (RT) which is predicted to make its RNase H domain resemble structurally that of human immunodeficiency virus RT. This deletion was based on improved RNase H homology alignments made possible by the recently solved three-dimensional structure for *Escherichia coli* RNase H. This mutant Moloney murine leukemia virus RT was fully active in the oligo(dT)-poly(rA) DNA polymerase assay and retained nearly all of wild-type RT's RNase H activity in an in situ RNase H gel assay. However, proviruses reconstructed to include this deletion were noninfectious. Minus-strand strong-stop DNA was made by the deletion mutant, but the amount of minus-strand translocation was intermediate to the very low level measured with RNase H-null virions and the high level seen with wild-type RT. The average length of translocated minus-strand DNA was shorter for the deletion mutant than for wild type, suggesting that mutations in the RNase H domain of RT also affect DNA polymerase activity.

Retroviral replication requires virally encoded reverse transcriptase (RT) to synthesize a double-stranded DNA copy of the genomic RNA (1, 11, 46). All retroviral RTs contain both a DNA polymerase activity, capable of synthesis on either RNA or DNA templates, and a nuclease activity, called RNase H, which specifically degrades RNA when the RNA is present in RNA:DNA hybrid form (27). Both of these activities are believed to be required for the completion of the complex series of steps involved in the RT-catalyzed biosynthesis of a double-stranded DNA copy of the retroviral RNA genome (10). RNase H has been implicated in several of these steps, and mutant viruses which lack RNase H activity are noninfectious (30, 34, 43, 47). Minus-strand strong-stop DNA, the first DNA intermediate to appear during reverse transcription, is maintained in RNA:DNA hybrid form among the abortive products of reverse transcription by virions which lack RNase H (43). This finding suggests that one of the first critical steps mediated by retroviral RNase H is the degradation of the RNA strand of this hybrid in order that minus-strand translocation and its subsequent elongation may occur.

Sequence comparisons reveal that RT is the most highly conserved protein among retroviruses, and sequence conservation exists between retroviral RT and proteins of other retroelements (9, 20, 25). Even related enzymes from cellular sources such as *Escherichia coli* and yeast RNases H can be aligned with the carboxy termini of retroviral RTs (51). However, the RTs of various retroviruses differ structurally in terms of subunit composition and organization. In the case of the Moloney murine leukemia virus (M-MuLV) enzyme, the DNA polymerase and RNase H activities reside in physically separable domains of a single monomeric protein (41). The human immunodeficiency virus (HIV) enzyme is a heterodimer whose subunits contain identical amino-terminal DNA polymerase regions, but only one subunit includes the carboxy-terminal RNase H region (8, 14, 23, 39). RT from the avian sarcoma-leukosis virus group, including Rous sarcoma virus RT, is also a heterodimer, but both subunits retain the DNA polymerase and RNase H domains and differ by the inclusion of the integrase region at the C terminus of one of the subunits.

The three-dimensional structure of *E. coli* RNase H was determined recently (21, 51). On the basis of this structure, improved homology alignments, which seek to preserve the secondary structure features and putative catalytic core geometry of *E. coli* RNase H, have been made between the *E. coli* enzyme and the RNase H domains of retroviral RTs. The validity of the resulting predicted retroviral RNase H structures has been partially confirmed by the recent elucidation of the structure of an HIV RT-derived polypeptide containing the region of RNase H homology (6). What emerges from these studies is a striking structural similarity among RNases H from evolutionarily distant sources. Although many RNases H differ in minor ways, such as in the number of residues between some of their secondary structure features, a more significant difference exists between HIV RNase H and the predicted structure for the M-MuLV RNase H domain. Tertiary structure predictions suggest that the RNase H domains of M-MuLV RT and *E. coli* RNase H are very similar structurally (51). Whereas model structures for both of these enzymes include five β strands and five α helices, one of the α helices is missing from HIV RNase H. In the experiments presented here, we have studied a mutant M-MuLV, which we call ΔC, with an 11-amino-acid deletion in RT which is predicted to make its RNase H domain resemble structurally that of HIV RT. The residues that we removed, 593 (Ile) through 603 (Leu) from M-MuLV RT, are predicted to include the α helix in the M-MuLV enzyme which is not present in HIV RT.

We have previously reported the effects of elimination of RNase H activity in the murine system by studying a series of mutants of M-MuLV with linker-insertion mutations in the RT region of the *pol* gene (41, 43). Unlike these previous

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RT mutants, whose RNase H activity is reduced profoundly or abolished, we demonstrate here that the ΔC mutant retained much of its basic RNase H activity but was nonetheless unable to sustain viral infectivity. In this report, we examine the replication defects of the ΔC mutant and expand our previous analyses of the effects of RNase H mutations on retroviral replication by establishing assays to compare the extents of minus-strand translocation by wild-type, ΔC, and RNase H-null forms of RT. We also report evidence which suggests that mutations in the RNase H domain of RT can affect elongation properties of the DNA polymerase activity of the enzyme without perturbing the basic catalytic activity of DNA polymerization, thus strengthening the notion that the DNA polymerase and RNase H activities of RT act together, both functionally and structurally, in retroviral replication.

MATERIALS AND METHODS

Cells and DNA-mediated transformations. All virus-producing cell lines were single-cell cloned from populations of NIH 3T3 cells which were subjected to calcium phosphate-mediated cotransformation with a mixture of proviral DNA and pSV2neo (38, 49). Transient transfection with DEAE-dextran, RT assays of supernatant medium, maintenance of cells in Dulbecco modified Eagle medium supplemented with 10% newborn calf serum, centrifugal concentration of viral particles, XC plaque assays, infection with virus in the presence of Polybrene, isolation of low-molecular-weight DNA, and Southern blot analysis were all performed as previously described (12, 15, 26, 32, 37, 42). The cell line used as a producer of virions containing wild-type RT was *dl*5401, which harbors a mutation in IN (36).

DNA manipulations. Subcloning into the *E. coli* RT expression vector (44) and into pNCA, which contains a cloned copy of the M-MuLV genome (3), and construction of M13 derivatives involved routine techniques (33). The *E. coli* expression vector contains the T7 phage A1 promoter and the sequences encoding mature M-MuLV RT preceded by a methionine codon (5, 40, 44, 45). Microsequencing of the purified wild-type RT produced by this vector revealed that the N-terminal methionine had been removed (data not shown). Thus, this expression system produces RT identical in sequence to that found in M-MuLV particles. ΔRH RT is a truncated form of M-MuLV RT; its construction will be described elsewhere (44). It produces a DNA polymerase single-domain form of RT which is missing the carboxy-terminal RNase H domain, and is similar to truncated forms of RT which have been described previously (16, 22, 41). The ΔC deletion was constructed by oligonucleotide-mediated site-directed mutagenesis (52). Annealing the oligonucleotide, 5′-CCTTCTGATGTGAGATGGGCAGTAGCAA-3′, to a single-stranded M13 derivative containing much of the M-MuLV RT coding region and then following standard site-directed mutagenesis procedures resulted in the loss of the 33 nucleotides (nt) which encode the 11 amino acids from 593 (Ile) through 603 (Leu) of M-MuLV RT.

Preparation and assay of enzymes. RT overexpressed in *E. coli* was purified essentially as we have described previously (31). All enzymes were purified from *E. coli* DH5α, which is a derivative of DH1 (13). All buffers and methods were the same, with the following modifications. Prior to passage through the DEAE-cellulose column, 0.6 volume of saturated ammonium sulfate (pH 7) was added to the crude extract supernatant. After 30 min of stirring at 4°C, the precipitated proteins were collected by centrifugation at 16,000 rpm for 30 min in a chilled SS34 rotor, gently resuspended in a small volume of buffer M (50 mM Tris-HCl [pH 7], 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 10% glycerol), and desalted by chromatography through a Sephadex G-50 column which had been equilibrated with buffer M containing 75 mM NaCl. Fractions that contained RT activity were pooled, passed through DEAE-cellulose, and then fractionated further by chromatography on phosphocellulose. The phosphocellulose column fractions containing the RT peak were pooled and concentrated in Centriprep 10 concentrators (Amicon) according to the manufacturer's instructions and dialyzed into storage buffer. This material was stored at −80°C for use in the experiments in this study. Estimations of purity, analyses of RT DNA polymerase activity using the oligo(dT)-poly(rA) assay, and unit definitions were as described previously (31).

RNase H activity was assessed by using an in situ gel assay as described previously (41). This assay involves electrophoresis of the proteins on sodium dodecyl sulfate-polyacrylamide gels cast with ^{32}P-labeled RNA in RNA:DNA hybrid form, followed by incubation of the gels in several changes of a buffer, which allows the renaturation of the protein and digestion of the radiolabeled hybrid. The gels are then fixed, dried, and exposed to X-ray film. Levels of RNase H activity are estimated by comparing the extent of radioactive clearing, which results from digestion of the labeled RNA.

Endogenous RT reactions. Endogenous reactions using concentrated virus were carried out for 3 h at 40°C in the presence of 100 μg of actinomycin D per ml and 2 mM each dCTP, dATP, and dGTP and 1 mM [α-^{32}P]dTTP at 1 Ci/mmol. These reactions were performed, and their products were processed and collected, as described previously (43). Reaction products were incubated in 0.33 N NaOH for 20 min at 55°C before the final ethanol precipitation to remove RNA primers. Reaction products were analyzed on 7.5% polyacrylamide gels, and DNA fragments were recovered from wet gels as previously described (43). Alkaline agarose electrophoresis was performed by standard methods (33).

Translocation assays. S1 assays for translocation made use of an unlabeled single-stranded M13 derivative containing the plus strand of a *Sac*I-*Sac*I fragment of pNCA, which includes 36 nt of U3 upstream of intact R and U5 regions of M-MuLV (3). This plus-strand DNA was hybridized to radiolabeled endogenous reaction products and digested with S1, and the labeled products were analyzed by using standard procedures for S1 mapping (33).

The restriction digest assay of translocation made use of a similar M13 derivative which contained the plus strand of a *Pst*I-*Eco*RI fragment of pNCA. This fragment includes intact U3, R, and U5 regions of the M-MuLV long terminal repeat (LTR). Portions of radiolabeled endogenous reactions estimated to contain less than 10 fmol of replication products were annealed to 200 fmol of the plus-strand DNA in 10-μl reaction mixtures containing 75 mM NaCl, 10 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.5) by incubating the reaction mixtures for 40 min at 66°C and then slowly cooling the mixtures to 25°C over 1 h. A 100-μl volume of a buffer containing 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 5 mM 2-mercaptoethanol, and 30 U *Ava*I was added to each sample, and the reaction mixtures were incubated at 37°C for 3 h. The products were then phenol extracted, ethanol precipitated, and analyzed by electrophoresis on 7.5% polyacrylamide–8 M urea gels. The gels were

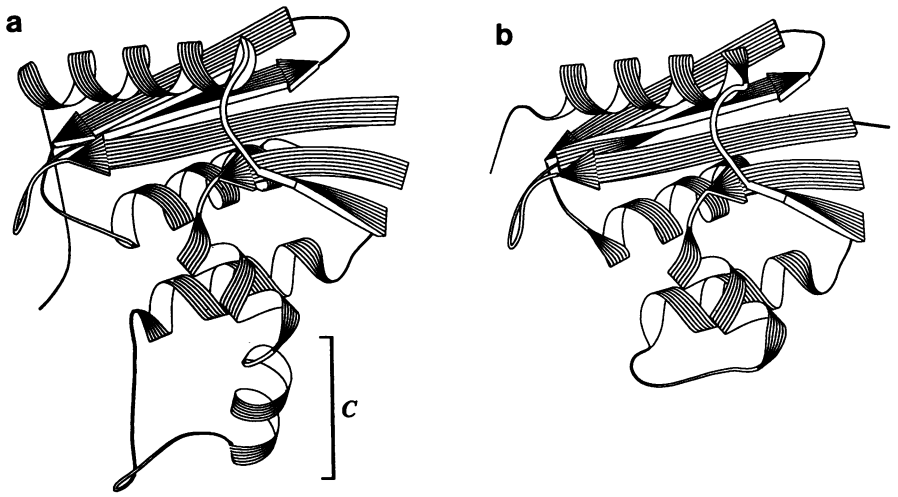

FIG. 1. Schematic ribbon drawings of the structure of *E. coli* RNase H (a) and a structural prediction for the HIV RNase H domain (b). The position of the C helix in the *E. coli* structure is indicated with a bracket. For more details, see reference 51.

dried and exposed to X-ray film with intensifying screens for 2 weeks at −80°C.

RESULTS

The C helix is not essential to basic RNase H function. Although the core structures of evolutionarily divergent RNases H are conserved, there are significant differences between the predicted structures of various retroviral RNases H. The third α helix present in the *E. coli* enzyme, known as the C helix (the first and second helices being designated A and B), is predicted to be present in the M-MuLV enzyme. However, it is missing from the HIV RNase H structure and also predicted to be absent from the dimeric RT of Rous sarcoma virus (Fig. 1 [6, 51]). This finding suggested that the C helix might be either dispensable for RNase H function or else responsible for some of the differences between such RNases H as those of M-MuLV and HIV. In the bacterial enzyme, the region of this deletion forms an α helix that is part of a flap of the enzyme which is not close to the catalytic site but which may contact the RNA:DNA hybrid when it is engaged at the active site (51). The absence of this helix in the HIV enzyme results in the flattening of this region of the protein relative to the structure of the *E. coli* enzyme. It has been suggested that this flattening allows the HIV RNase H domain to interact with some portion of the two DNA polymerase domains of the intact heterodimer and that this interaction both stabilizes the dimer and is essential for productive substrate binding by the RNase H domain (6).

The M-MuLV RT mutant in this study is called ΔC because it has an 11-amino-acid deletion in the enzyme's RNase H domain which removed a region predicted to include the C helix. To create the ΔC mutant, we examined the published structure of *E. coli* RNase H and homology alignments made between it and retroviral RNase H regions and then chose to delete residues 593 (Ile) through 603 (Leu) of M-MuLV RT. The ΔC deletion was constructed by oligonucleotide-mediated site-directed mutagenesis and built into an *E. coli* expression plasmid encoding authentic M-MuLV RT (33, 44, 52). Wild-type and ΔC M-MuLV RT and a truncated form of the enzyme called ΔRH, which is missing the entire RNase H domain, were purified to >90% homogeneity from *E. coli* cells harboring RT expression plasmids by a variation of the method of Roth et al. (31). The DNA polymerase activities of these enzymes were compared by measuring incorporation of TMP, using an oligo(dT)-poly(rA)primer-template. Wild-type, ΔC, and ΔRH RT were determined to display 44, 99, and 112 U of DNA polymerase activity per μg of protein, respectively. Thus, according to this assay, the DNA polymerase activity of these RT mutants is not reduced; rather, it is higher than that of the wild-type enzyme.

RNase H activities were compared in an in situ gel assay (41). The amounts of degradation of RNA:DNA hybrid by 10, 20, and 40 U of DNA polymerase activity of each enzyme were compared (Fig. 2). By this comparison, it appears that the ΔC enzyme retains all or nearly all of the RNase H activity of the wild-type enzyme. Thus, whereas the ΔC mutation results in the removal of the central 1/10 of the RNase H domain, the catalytic site has not been destroyed, and we have successfully constructed a largely functional RNase H allele of RT based on the crystal structure of the related enzyme, *E. coli* RNase H.

Note that we were unable to quantify our partially purified

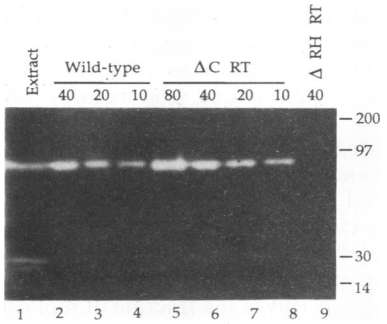

FIG. 2. In situ gel assay for RNase H activity. Proteins purified from *E. coli* RT expression strains were quantified as described in Materials and Methods; lane 1 contains wild-type RT in a crude extract prepared as described by Hizi et al. (17). Numbers at the top indicate the number of units of RT DNA polymerase activity loaded in each lane. Sizes in kilodaltons are indicated at the right.

enzymes' RNase H activities in solution, since these enzyme preparations may contain some residual nucleases. Extracts from *E. coli* cells contain several host-encoded RNase H activities. Whereas no contaminating *E. coli* RNase H activities were detectable in our purified enzyme preparations in the in situ assay, solution assays suggested that our ΔRH RT contains significant levels of contaminating RNase H activity (data not shown).

Viruses harboring the ΔC mutation are noninfectious. The ΔC mutation was moved into a cloned copy of the M-MuLV genome, and the infectivity of the resulting mutant provirus was tested in 3T3 cells. No viral spread was detectable, either by the XC plaque assay or by assaying culture media for RT, when the cells were transiently transfected with this provirus via DEAE-dextran.

Cell lines that produced RT^+ virions harboring the ΔC mutation were established. When virions shed by these cell lines were applied to fresh 3T3 cells, they were found to be noninfectious, and no low-molecular-weight (preintegrative) viral DNA was detected on Southern blots (data not shown). No reversion of the ΔC mutation was observed during 6 weeks of passage of cells infected with ΔC virions. Therefore, we conclude that the ΔC mutation renders M-MuLV noninfectious.

Minus-strand translocation is reduced in ΔC virions. To assess the nature of the replication defects conferred by the ΔC mutation, we compared the replication properties of ΔC virions with those of virions with wild-type RT and two previously described RNase H-null forms of RT (41, 43). One of the M-MuLV RT RNase H null alleles (M4) is a linker-insertion mutation which destroys RNase H activity, and the other (H7) contains a frameshift early in the RNase H coding region which also abolishes RNase H activity.

Both the wild type and all of the RNase H mutants formed minus-strand strong-stop DNA when replication products were examined by using detergent-permeabilized virions in the so-called endogenous reaction (Fig. 3). However, the amounts of slower-migrating products of the endogenous reaction were significantly reduced for all of the RNase H mutants. Since these reactions were performed in the presence of actinomycin D, which prevents DNA-dependent DNA synthesis, these slower-migrating DNAs should be limited to minus-strand products.

Since RNase H activity is believed to be involved in the translocation of minus-strand strong-stop DNA to the 3′ end of the genomic RNA (necessary for the completion of minus-strand DNA synthesis), we developed two assays to assess the extent of translocation (Fig. 4). One of these was an S1 protection assay which allowed determination of the extent of minus-strand translocation by comparing the ratio of minus-strand products whose synthesis ceased at the stage of minus strand strong stop to those which had been extended after translocation and thus included sequences from the U3 region. The second assay involved cleavage of the labeled endogenous reaction products hybridized to single-stranded plus-strand LTR DNA by a restriction enzyme which cuts in both the U5 and U3 regions. This analysis yielded products a and b for minus-strand strong-stop DNA and products a and b′ for translocated DNA, and the ratio b′/(b + b′) gives the fraction of replication products that had translocated (Fig. 4).

These assays revealed that the null alleles of RNase H translocate to a very low but detectable extent and that the extent of translocation of minus-strand strong-stop DNA by the ΔC enzyme is intermediate to that observed with the wild-type and mutant enzymes. Translocation by the RNase

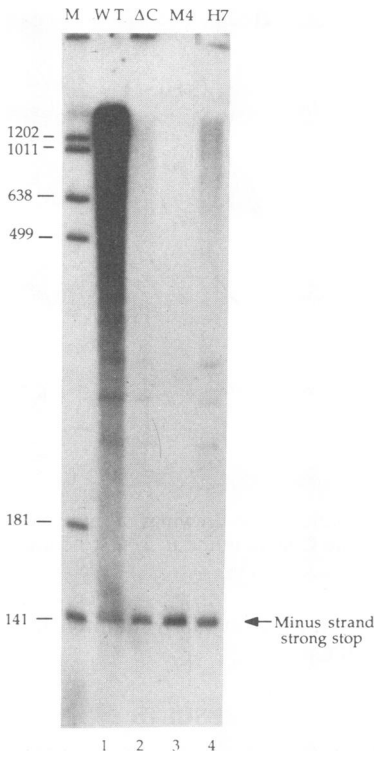

FIG. 3. Endogenous reaction products from wild-type and RNase H mutant virions analyzed by polyacrylamide gel electrophoresis. WT indicates the products from virions containing wild-type RT; ΔC, M4, and H7 are the products of virions with RNase H mutations which are described in the text. The position of migration of minus-strand strong-stop DNA is indicated. Marker sizes are in nucleotides. Note that the discrete bands migrating between the 181- and 499-nt markers are VL30 minus-strand strong-stop DNAs which are produced from copackaged viruslike RNAs (43).

H-null forms of RT could not be detected when the assays were performed on total reaction products. Thus, to assess whether any minus-strand translocation had occurred, we fractionated the endogenous reaction products of an RNase H-null allele. DNAs were eluted from gel slices containing populations of high-molecular-weight reaction products. The isolated DNAs called ii, iii, and iv are populations of molecules approximately 500 to 800, 800 to 1,200, and >1,200 nt long, respectively (Fig. 4D). When these isolated DNAs were assayed, very low levels of translocation were detectable. We estimate that translocation by the null alleles is lower than for the wild type by 2 or 3 orders of magnitude; in contrast, translocation by ΔC is approximately 10-fold reduced.

The S1 protection assay of ΔC endogenous reaction products also revealed a prominent set of DNA products less than 40 nt in length which were protected by plus-strand LTR DNA (Fig. 4b). The size and mapping properties of these products suggest that they are prematurely terminated tRNA-initiated minus-strand products. As is visible in Fig. 5, there are prominent DNAs smaller than minus-strand strong-stop DNA among the ΔC endogenous reaction products that may contribute to these protected products.

Translocated minus-strand products of ΔC are shorter than those of the wild type. The amount of longer minus-strand products in the ΔC reaction appeared less than expected for the measured amount of translocation, and the gel system

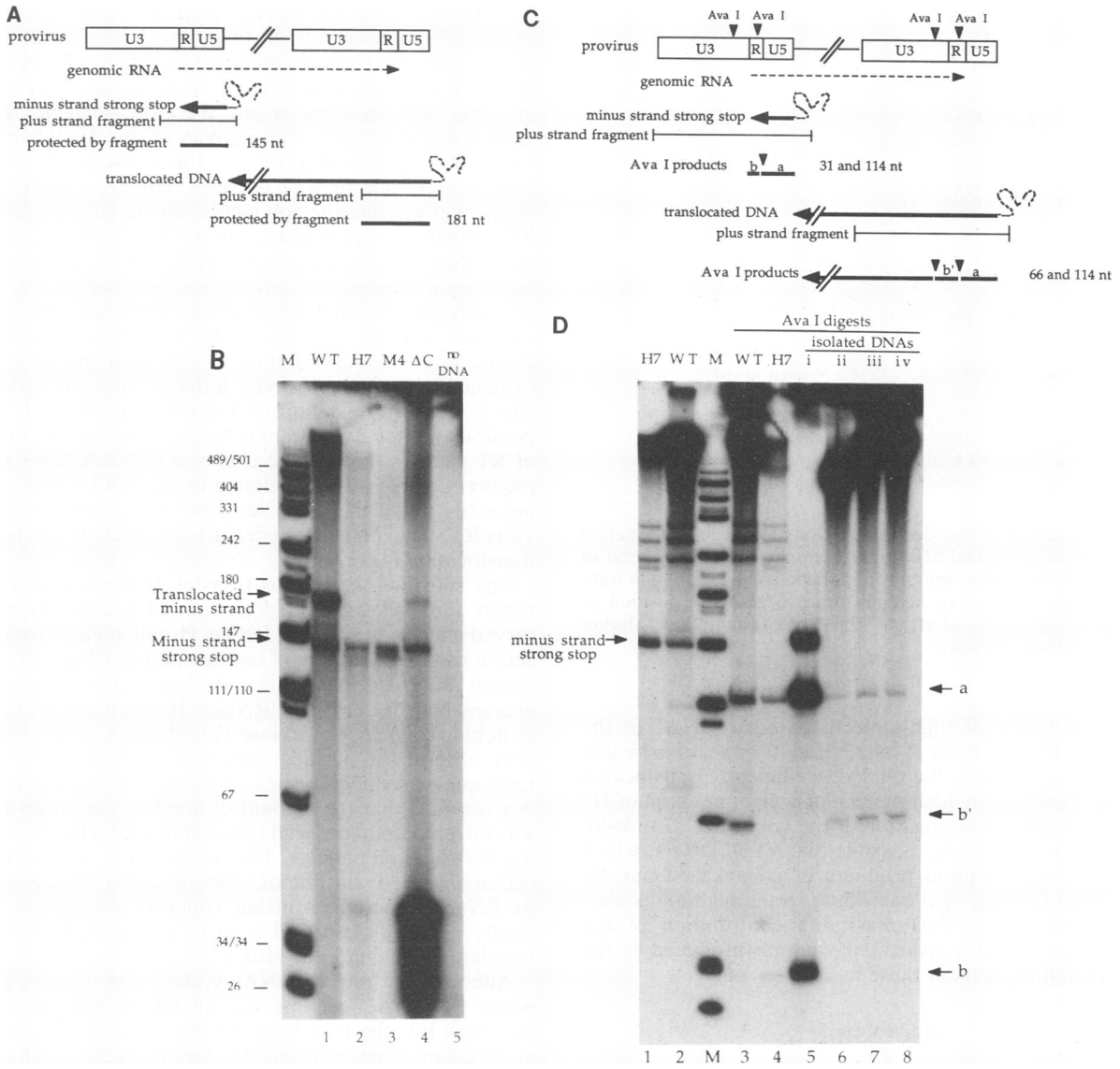

FIG. 4. Translocation assays. (A) Schematic explanation of the S1 assay for translocation. Dotted lines indicate RNA, solid lines indicate DNA, and heavy lines indicate DNA radiolabeled during its synthesis in endogenous reactions. (B) Products of the S1 assay. Endogenous reaction products hybridized to plus-strand DNA are indicated above the lanes. The positions of migration of digestion products generated from minus-strand strong-stop and translocated minus-strand DNAs are indicated. Marker sizes are in nucleotides. (C) Schematic explanation of the restriction digest assay of translocation. Dotted lines indicate RNA, solid lines indicate DNA, and heavy lines indicate DNA radiolabeled during its synthesis in endogenous reactions. (D) Products of the restriction digest assay. Lanes 1 and 2 contain undigested endogenous reaction products from virions containing the RNase H mutant H7 or wild-type form of RT. Lanes 3 and 4 contain the products of the restriction digest assay performed on unfractionated endogenous reaction products. Lanes 5 to 8 contain the products of restriction digest assays performed on DNAs isolated from wet gels containing RNase H mutant H7 endogenous reaction products; the isolated DNA labeled i is purified minus-strand strong-stop DNA; DNAs ii, iii, and iv are populations of molecules which were eluted from gel slices of successively higher molecular weight endogenous reaction products. Lanes 5 to 8 contain approximately equivalent portions of endogenous reaction products; the amounts of endogenous reaction products assayed in lanes 5 to 8 are much greater than those in lanes 1 to 4 so that minor translocation products could be detected. The positions of migration of the restriction digest fragments and of intact minus-strand strong-stop DNA are indicated; the markers are the same as those in panel B.

used in the assays described above was not capable of resolving the sizes of large reaction products. Thus, it seemed possible that ΔC virions generated translocated reaction products shorter than those produced in virions harboring wild-type RT. To compare the sizes of translocated minus-strand products, aliquots containing similar levels of radioactivity from ΔC and wild-type endogenous reactions were analyzed by denaturing agarose gel electrophoresis (Fig. 5). Whereas a significant level of DNA migrating in the vicinity of the predicted size for fully extended minus-strand products was observed in reactions containing wild-type RT, translocated ΔC products averaged less than one-third this length.

Comparison of the products of mutants ΔC and H7 (Fig. 3) shows that even though the efficiency of translocation during reverse transcription is very different, these viruses make

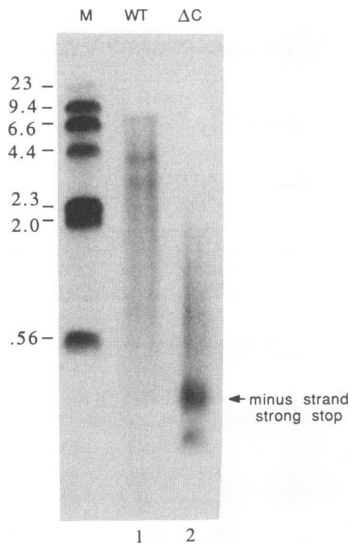

FIG. 5. Analysis of the size distribution of endogenous reaction products of wild-type and ΔC virions. Samples were normalized so that approximately equal amounts of radioactive incorporation were loaded in each lane of an alkaline agarose gel. The position of migration of minus-strand strong-stop DNA is indicated. Marker sizes are in kilobase pairs.

similar low levels of high-molecular-weight DNA. In the case of ΔC, the low level of long products appears to be due to limited elongation of the relatively efficiently translocated products. Perhaps the high-molecular-weight material of H7 results from efficient elongation of the very rare H7 translocation products. It is also possible that VL30 DNAs, which are reverse transcription products of copackaged non-M-MuLV RNAs, might also contribute to the high-molecular-weight material and thus mask the contribution of the miniscule amount of translocated products produced by the RNase H-null mutants in these assays.

DISCUSSION

On the basis of the crystal structure of *E. coli* RNase H, we have constructed a deletion mutation called ΔC in the RNase H domain of M-MuLV RT. The studies presented here were aimed at characterizing the phenotype of the ΔC mutant. The late stages of the life cycle were apparently normal for the mutant. Cell lines expressing the ΔC mutant release virions containing genomic RNA and RT activity, and examination of these virions by immunoprecipitation of metabolically labeled proteins showed that the *gag* precursor was processed correctly (data not shown). However, viruses containing this mutation were noninfectious when applied to cells and were incapable of producing detectable amounts of preintegrative DNA. The very low titer of ΔC virus has prevented a direct analysis of the processing of ΔC *pol* products. However, since correct *pol* processing occurs in the RNase H deletions that we have previously studied, we believe that the primary defect of ΔC is probably not in the processing of RT from its precursor (43).

Unlike our previous RNase H linker-insertion mutants, the ΔC enzyme, which is missing the central 1/10 of its RNase H domain, retained much of its basic RNase H activity. It seems unlikely that the lethality of ΔC is due solely to the relatively modest decrease in basic RNase H activity that this mutation caused. It has been estimated that there are approximately 50 times more RT molecules than genomic RNAs in virions (48), and the DNA polymerase and RNase H activities of RT can be shown to act independently in some settings. One might therefore imagine that the inefficiency of the RNase H activity of one ΔC RT molecule could be compensated for by the subsequent action of another molecule of RT. There is, however, some controversy over whether the polymerase and nuclease activities of RT normally do act in concert in in vitro reactions, and even less is known about the potentially obligate coupling of these activities during normal retroviral replication (7, 19, 28, 35, 50). It is possible that ΔC might be affected in the putative coupling of these two enzymatic activities. Alternatively, even a subtle defect in RNase H activity might be sufficient to account for the observed lethality, due to cumulative effects during replication of the entire genome. It is also possible that the ΔC mutant is defective in specific functions of RT such as the specific cleavage of RNA:DNA hybrids required for plus-strand priming or the RNA-DNA unwinding activity that has been described for avian myeloblastos virus RT, which may be necessary for efficient minus-strand translocation (4).

The sensitive assays for translocation presented in this report show that a limited amount of translocation is observed even in the case of RNase H-null alleles. Though the action of trace amounts of contaminating RNase H activity cannot be ruled out, this low level of translocation is presumably due to the RNase H-independent template switching capability of RNase H-null forms of RT which has been observed in purified systems (24). Examination of the replication products formed in purified ΔC-containing virions revealed that the amount of minus-strand translocation was lower than that of wild type but much higher than that of the RNase H-null alleles. The much higher level of translocation observed with the ΔC mutant, which retains much of its RNase H activity, further supports the belief that the virally encoded RNase H activity is required for efficient template switching in M-MuLV.

Although the rate of DNA synthesis on exogenous templates by the ΔC enzyme was slightly higher than that of wild-type RT, the translocated minus-strand products made in detergent-permeabilized ΔC virions were much shorter than those formed in virions harboring wild-type RT, thus suggesting that minus-strand synthesis terminated prematurely. Additionally, experiments with purified enzymes have shown that the ΔC and ΔRH enzymes are defective in processivity of DNA synthesis (44). In template competition experiments, the mutant enzymes produce shorter template-directed products than the wild-type enzyme does (data not shown). Thus, the block to replication in the ΔC mutation may result from subtle deficiencies in the DNA polymerase activities of the enzyme as much as from deficiencies in the RNase H activity itself.

We are currently constructing additional mutations in the RNase H domain of M-MuLV RT to examine the importance of regions which vary among the retroviral RNases H and to test predictions, based on the crystal structure, of the properties associated with various portions of the enzyme. Studying other C-helix mutations may further define the role of this region. Although the C helix is not required for basic RNase H activity in intact M-MuLV RT, its absence may be permissible because of structural information provided by the DNA polymerase domain. Wild-type M-MuLV RNase H, with the C helix intact, is very active when expressed as a single domain in *E. coli* (41). However, when the ΔC

mutation was rebuilt into an RNase H single-domain expression vector, no RNase H activity was observed in in situ gel assays, which should detect activity as low as 1% of the wild-type level (2). Since ΔC was designed to resemble HIV RNase H, this provides an interesting parallel to the difficulties that our laboratory and other laboratories have encountered in attempts to produce an active single-domain HIV RNase H (6, 16–18, 29).

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REFERENCES

1. **Baltimore, D.** 1970. RNA-dependent DNA polymerase in virions of RNA tumor viruses. Nature (London) **226**:1209–1211.
2. **Blain, S. W., A. Telesnitsky, and S. P. Goff.** Unpublished data.
3. **Colicelli, J., and S. P. Goff.** 1988. Sequence and spacing requirements of a retrovirus integration site. J. Mol. Biol. **199**:47–59.
4. **Collett, M. S., J. P. Leis, M. S. Smith, and A. J. Faras.** 1978. Unwinding-like activity associated with retrovirus RNA-directed DNA polymerase. J. Virol. **26**:498–509.
5. **Copeland, T. D., G. F. Gerard, C. W. Hixson, and S. Oroszlan.** 1985. Amino- and carboxyl-terminal sequence of Moloney murine leukemia virus reverse transcriptase. Virology **143**:676–679.
6. **Davies, J. F. I., Z. Hostomska, S. R. Jorden, and D. A. Matthews.** 1991. Crystal structure of the ribonuclease H domain of HIV-1 reverse transcriptase. Science **252**:88–95.
7. **DeStefano, J. J., R. G. Buiser, L. M. Mallaber, T. W. Myers, R. A. Bambara, and P. J. Fay.** 1991. Polymerization and RNase H activities of the reverse transcriptases from avian myeloblastosis, human immunodeficiency and Moloney murine leukemia viruses are functionally uncoupled. J. Biol. Chem. **266**:7423–7431.
8. **di Marzo Veronese, F. D., T. D. Copeland, A. L. DeVico, R. Rahman, S. Oroszlan, R. C. Gallo, and M. G. Sarngadharan.** 1986. Characterization of highly immunogenic p66/p51 as the reverse transcriptase of HTLV-III/LAV. Science **231**:1289–1291.
9. **Doolittle, R. F., D.-F. Feng, M. S. Johnson, and M. A. McClure.** 1989. Origin and evolutionary relationships of retroviruses. Q. Rev. Biol. **64**:1–30.
10. **Gilboa, E., S. W. Mitra, S. P. Goff, and D. Baltimore.** 1980. A detailed model of reverse transcription and tests of crucial aspects. Cell **18**:93–100.
11. **Goff, S. P.** 1990. Retroviral reverse transcriptase: synthesis, structure and function. J. AIDS **3**:817–831.
12. **Goff, S. P., 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.
13. **Hanahan, D.** 1983. Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. **166**:557–580.
14. **Hansen, J., T. Schulze, W. Mellert, and K. Moelling.** 1988. Identification and characterization of HIV-specific RNAse H by monoclonal antibody. EMBO J. **7**:239–243.
15. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
16. **Hizi, A., S. H. Hughes, and M. Shararabany.** 1990. Mutational analysis of the ribonuclease H activity of human immunodeficiency virus reverse transcriptase. Virology **175**:575–580.
17. **Hizi, A., C. McGill, and S. H. Hughes.** 1988. Expression of soluble, enzymatically active human immunodeficiency virus reverse transcriptase in *Escherichia coli* and analysis of mutants. Proc. Natl. Acad. Sci. USA **85**:1218–1222.
18. **Hostomsky, Z., Z. Hostomska, G. O. Hudson, E. W. Woomaw, and B. R. Nodes.** 1991. Reconstitution *in vitro* of RNase H activity by using purified N-terminal and C-terminal domains of human immunodeficiency virus type 1 reverse transcriptase. Proc. Natl. Acad. Sci. USA **88**:1148–1152.
19. **Huber, H. E., J. M. McCoy, J. S. Seehra, and C. C. Richardson.** 1989. Human immunodeficiency virus 1 reverse transcriptase. J. Biol. Chem. **264**:4669–4678.
20. **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 enzymes. Proc. Natl. Acad. Sci. USA **83**:7648–7652.
21. **Katayanagi, K., M. Miyagawa, M. Matsushima, M. Ishikawa, S. Kanaya, M. Ikehara, T. Matsuzaki, and K. Morikawa.** 1990. Three-dimensional structure of ribonuclease H from *E. coli*. Nature (London) **347**:306–309.
22. **Kotewicz, M. L., C. M. Sampson, J. M. D'Alessio, and G. F. Gerard.** 1988. Isolation of cloned Moloney murine leukemia virus reverse transcriptase lacking ribonuclease H activity. Nucleic Acids Res. **16**:265–277.
23. **Lightfoote, M. M., J. E. Coligan, T. M. Folks, A. S. Fauci, M. A. Martin, and S. Venkatesan.** 1986. Structural characterization of reverse transcriptase and endonuclease polypeptides of the acquired immunodeficiency syndrome retrovirus. J. Virol. **60**:771–775.
24. **Luo, G. X., and J. Taylor.** 1990. Template switching by reverse transcriptase during DNA synthesis. J. Virol. **64**:4321–4328.
25. **McClure, M. A., M. S. Johnson, D.-F. Feng, and R. F. Doolittle.** 1988. Sequence comparisons of retroviral proteins: relative rates of change and general phylogeny. Proc. Natl. Acad. Sci. USA **85**:2469–2473.
26. **McCutchan, J. H., and J. S. Pagano.** 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. J. Natl. Cancer Inst. **41**:351–357.
27. **Moelling, K., D. P. Bolognesi, W. Bauer, W. Busen, H. W. Passman, and P. Hausen.** 1971. Association of viral reverse transcriptase with an enzyme degrading the RNA moiety of RNA:DNA hybrids. Nature (London) New Biol. **234**:240–243.
28. **Oyama, F., R. Kikuchi, R. J. Crouch, and T. Uchida.** 1989. Intrinsic properties of reverse transcriptase in reverse transcription; associated RNase H is essentially regarded as an endonuclease. J. Biol. Chem. **264**:18808–18817.
29. **Prasad, V. R., and S. P. Goff.** 1989. Linker insertion mutagenesis of human immunodeficiency virus reverse transcriptase expressed in bacteria: definition of the minimal polymerase domain. Proc. Natl. Acad. Sci. USA **86**:3104–3108.
30. **Repaske, R., J. W. Hartley, M. F. Kavlick, R. R. O'Neill, and J. B. Austin.** 1989. Inhibition of RNase H activity and viral replication by single mutations in the 3′ region of Moloney murine leukemia virus reverse transcriptase. J. Virol. **63**:1460–1464.
31. **Roth, M., 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.
32. **Rowe, W. P., W. E. Pugh, and J. W. Hartley.** 1970. Plaque assay techniques for murine leukemia viruses. Virology **42**:1136–1139.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Schatz, O., F. Cromme, F. Grueninger-Leitch, and S. F. J. Le Grice.** 1989. Point mutations in conserved amino acid residues within the C-terminal domain of HIV-1 reverse transcriptase specifically repress RNase H function. FEBS Lett. **257**:311–314.
35. **Schatz, O., J. Mous, and S. F. Le Grice.** 1990. HIV-1 RT-associated ribonuclease H displays both endonuclease and 3′-5′ exonuclease activity. EMBO J. **9**:1171–1176.
36. **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 proviral function required for the establishment of integrated provirus. Cell **37**:1043–1052.

37. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
38. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
39. **Starnes, M. C., and Y. C. Cheng.** 1989. Human immunodeficiency virus reverse transcriptase-associated RNase H activity. J. Biol. Chem. **264**:7073–7077.
40. **Studier, F. W., A. H. Rosenberg, M. N. Simon, and J. J. Dunn.** 1979. Genetic and physical mapping of the early region of bacteriophage T7 DNA. J. Mol. Biol. **135**:917–937.
41. **Tanese, N., and S. P. Goff.** 1988. Domain structure of the Moloney MuLV reverse transcriptase: mutational analysis and separate expression of the polymerase and RNAse H activities. Proc. Natl. Acad. Sci. USA **85**:1777–1781.
42. **Tanese, N., M. Roth, and S. P. Goff.** 1986. Analysis of retroviral *pol* gene products with antisera raised against fusion proteins expressed in *Escherichia coli*. J. Virol. **59**:328–340.
43. **Tanese, N. T., A. Telesnitsky, and S. P. Goff.** 1991. Abortive reverse transcription by mutants of Moloney murine leukemia virus deficient in the reverse transcriptase-associated RNase H function. J. Virol. **65**:4387–4397.
44. **Telesnitsky, A., and S. P. Goff.** Unpublished data.
45. **Telesnitsky, A. P. W., and M. J. Chamberlin.** 1989. Sequences linked to prokaryotic promoters can affect the efficiency of downstream termination sites. J. Mol. Biol. **205**:315–330.
46. **Temin, H. M., and S. Mizutani.** 1970. RNA-directed DNA polymerase in virions of Rous sarcoma virus. Nature (London) **226**:1211–1213.
47. **Tisdale, M., T. Schulze, B. A. Larder, and K. Moelling.** 1991. Mutations within the RNase H domain of human immunodeficiency virus type 1 reverse transcriptase abolish virus infectivity. J. Gen. Virol. **72**:59–66.
48. **Varmus, H., and P. Brown.** 1989. Retroviruses, p. 53–108. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington D.C.
49. **Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel.** 1979. Transformation of mammalian cells with genes from procaryotes and eucaryotes. Cell **16**:777–785.
50. **Wohrl, B. M., and K. Moelling.** 1990. Interaction of HIV-1 ribonuclease H with polypurine tract containing RNA-DNA hybrids. Biochemistry **29**:10141–10147.
51. **Yang, W., W. A. Hendrickson, R. J. Crouch, and Y. Satow.** 1990. Structure of ribonuclease H phased at 2 Å resolution by MAD analysis of the selenomethionyl protein. Science **249**: 1398–1405.
52. **Zoller, M. J., and M. Smith.** 1982. Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA. Nucleic Acids Res. **10**:6487–6500.