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Mechanism of RNA Primer Removal by the RNase H Activity of Avian Myeloblastosis Virus Reverse Transcriptase

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The single-stranded DNA containing the Moloney murine leukemia virus origin for plus-strand synthesis was cloned in M13mp2 and used as a template for avian myeloblastosis virus reverse transcriptase in the presence of Moloney RNA which had been treated with pancreatic RNase A. The RNA pieces containing the polypurine stretch near the plus-strand origin were processed, presumably by RNase H, to generate primers for DNA synthesis which initiated both at the correct origin site and at one nucleotide downstream from the correct site. Approximately 50% of the labeled DNA fragments synthesized under these conditions retained the priming RNA on their 5′ ends. When the isolated fragments were hybridized back to the template DNA and again treated with the reverse transcriptase, all of the RNA was removed from the labeled DNA. By using 5′-end-labeled pancreatic RNase A-resistant fragments, it was possible to show that the RNA primers were removed intact. It appears from these results that the RNase H activity associated with the enzyme shows a preference for cutting at the junction between the RNA and DNA moieties of such complexes and therefore is ideally suited for removing RNA primers.

Copying of retrovirus genome RNA into double-stranded DNA is accomplished by detergent-treated virions, with the enzyme reverse transcriptase playing the major and possibly sole role (8, 25). Many of the essential features of the reverse transcription reaction have been elucidated (2, 9, 12, 22, 24), but one aspect of the process requiring further investigation is initiation of the plus or second strand of DNA.

Reverse transcriptase contains a ribonuclease H activity as an integral part of its structure (1, 10, 16, 25). This enzyme degrades the RNA moiety of RNA-DNA hybrids and is therefore thought to degrade genome RNA after it has served as a template for the synthesis of minus-strand DNA (4, 5, 7, 26). The enzyme could, however, have other activities.

Based on current models of reverse transcription, there are two reactions other than genome degradation which might be catalyzed by an RNase H activity. First, because the tRNA primer is ultimately used as a template by reverse transcriptase to make plus-strand DNA, the RNase H activity could function to remove the primer RNA. Recent results indicate that RNase H can remove the tRNA primer from minus-strand DNA (19). Any RNA primers involved in the synthesis of plus-strand DNA could be similarly removed. Second, to assist the initiation of plus strands of DNA the RNase H activity could create the proper primer RNA by selective degradation of the RNA template (17, 18, 26). Recent evidence indicates that the site of initiation of plus strands is highly specific (14, 15), so that if an RNA primer is generated by this mechanism, the enzyme must recognize the correct sequence and precisely cleave the RNA. The experiments detailed here were initiated with the aim of determining the possible involvement of the RNase H in these two aspects of the process of reverse transcription.

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MATERIALS AND METHODS

Materials. Clone 1 Moloney murine leukemia virus (M-MuLV) (6) was grown and isolated as previously described (8). Purified avian myeloblastosis virus (AMV) reverse transcriptase (11) was kindly provided by J. Beard. All restriction enzymes and polynucleotide kinase were obtained from New England Biolabs. Radioactively labeled nucleotides were purchased from New England Nuclear Corp.

Nucleic acids. M-MuLV proviral DNA which had been cloned in pBR322 (21) was cut out with *Hin*dIII, followed by *Sac*I, and the three resulting fragments were recloned in pBR322, using *Eco*RI linkers. The plasmid containing the largest fragment (3.2 kilobase pairs), corresponding to the right third of the M-MuLV genome, was cut with *Eco*RI and cloned in M13mp2. By screening for phage DNAs which annealed with each other, both polarities of the inserted fragment were obtained. The plus-strand-containing recombinant phage was identified by the ability of the DNA to hybridize to labeled minus strands synthesized in detergent-treated virions in the presence of actinomycin D. The phage containing the complementary or minus-strand DNA (M13mp2/molA−) was used in the experiments described here. M13mp2 phage and recombinant derivatives were concentrated by precipitation with 2% polyethylene glycol 6000 in the presence of 0.5 M NaCl. The phage pellets were resuspended in 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA, made 0.1% in sodium dodecyl sulfate, extracted twice with phenol and twice with $CHCl_3$-isoamyl alcohol (24:1), and ethanol precipitated.

Authentic plus-strand strong-stop DNA was isolated from the endogenous reverse transcription reaction, cut with *Pvu*II, and 5′-end labeled as described previously (14).

RNA was extracted from M-MuLV by resuspending the virus in 20 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA, adding sodium dodecyl sulfate to 0.5%, and extracting the mixture twice with phenol-chloroform (1:1) and twice with chloroform. The RNA was precipitated with two volumes of ethanol and stored at −20°C.

Reverse transcriptase reaction conditions. The standard reaction mixture (20 μl) contained 50 mM Tris-hydrochloride (pH 8.3), 10 mM $MgCl_2$, 50 mM KCl, 0.4 mM dithiothreitol, 0.2 mM each of the unlabeled dNTPs, 20 to 30 μM ^{32}P-labeled dNTP, 20 to 40 μg of template DNA per ml, and the indicated units (11) of AMV reverse transcriptase. The reactions were incubated at 37°C for 60 min and terminated by heating to 65°C for 5 min, and the products were treated with 0.5 U of *Pvu*II for 60 min at 37°C. In some cases the RNA to be added to the reaction was pretreated with pancreatic RNase A at 1 μg/ml for 20 min in the complete reaction minus the template DNA, dNTPs, and reverse transcriptase.

Preparation of 5'-end-labeled M-MuLV genome RNase A-resistant fragments. M-MuLV virion RNA (1.2 μg) was treated with pancreatic RNase A (1 μg/ml) in the standard reverse transcriptase reaction minus the DNA and dNTPs for 20 min at 37°C. EDTA was added to 10 mM, 0.1 μg of simian virus 40 (SV40) DNA was added as a carrier, ammonium acetate was added to 2 M, and the mixture was precipitated with two volumes of ethanol. The ethanol precipitate was dried and then resuspended in 10 mM Tris-hydrochloride (pH 7.5), and the 5' ends were phosphorylated, using polynucleotide kinase and [γ-^{32}P]ATP under the conditions recommended by the supplier. The unincorporated label was removed by two ethanol precipitations in the presence of 2 M ammonium acetate.

Polyacrylamide gel electrophoresis. The products of the reverse transcriptase reactions were analyzed by electrophoresis in 5% polyacrylamide gels run in a Tris-borate-EDTA buffer (pH 8.3) as previously described (15). Before analysis, samples were ethanol precipitated, redissolved in 80% formamide containing 5 mM EDTA, and denatured by heating to 100°C for 2 min. Electrophoresis was for 2 to 2.5 h at the indicated voltages. The gels were dried on Whatman 3MM paper and then subjected to autoradiography. ^{32}P-labeled SV40 *Hin*fI fragments were used as markers.

Sequencing gels of 8 and 20% polyacrylamide containing 8 M urea were run according to published procedures (13).

Polyethyleneimine cellulose thin-layer chromatography. For ^{32}P transfer experiments, samples were hydrolyzed in alkali, mixed with marker ribonucleotide monophosphates, and applied to a 0.1-mm cellulose MN 300 polyethyleneimine plate which had been prerun with water and washed with methanol. The plate was developed at 5°C with water to the origin and then with 0.55 M $(NH_4)_2SO_4$ to the top. The radioactively labeled nucleoside monophosphates were located by autoradiography and compared with the four marker nucleotides identified with a UV light.

Other procedures. DNA samples were annealed for 40 min at 65°C in 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA–0.25 M NaCl and ethanol precipitated before use as substrates for the reverse transcriptase. DNA fragments were exposed to 0.30 M NaOH at 65°C for 20 min to hydrolyze any attached RNA. Mild alkaline hydrolysis was performed in sealed capillary tubes in 50 mM $NaHCO_3$ (pH 9.1) at 90°C for the indicated times.

RESULTS

Priming of DNA synthesis near the plus-strand origin. The origin of plus-strand RNA synthesis is preceded by a string of 13 purines. Since the first two nucleotides in the plus strand are also purines (A residues), treatment of M-MuLV virion RNA with pancreatic RNase should leave a 16-nucleotide-long fragment that contains these 15 purines plus a 3' terminal U residue. This RNase-resistant fragment should span the origin of plus-strand synthesis (Fig. 1). The presence of a 3'-phosphate group on this fragment should preclude its use as a primer by reverse transcriptase unless some activity in the reverse transcriptase preparation is capable of removing either the terminal phosphate or one or more nucleotides to produce a free 3'-hydroxyl group.

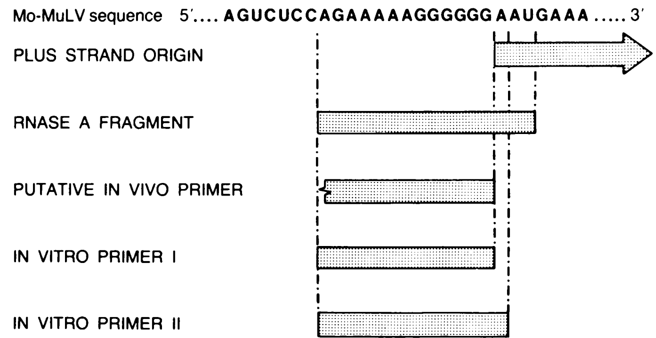

FIG. 1. Features surrounding the origin for plus-strand synthesis. The sequence of the M-MuLV genome in the vicinity of the plus-strand origin is represented along the top. Plus strands are known to originate at the sequence AATG . . . (shown by the arrow on the second line). The 16-nucleotide-long fragment which is resistant to pancreatic RNase A is indicated on the third line. The fourth line shows the structure expected for a putative RNA primer that could initiate plus DNA strands. The last two lines show the plus-strand primers that are generated by the reverse transcriptase from the RNase A-resistant fragment (see the text).

The M-MuLV minus-strand template containing the origin for plus-strand synthesis was cloned in M13mp2 and tested in a reaction with AMV reverse transcriptase in the presence of ^{32}P-labeled dCTP with intact or pancreatic RNase A-treated M-MuLV RNA as a potential primer. The products of the reaction were cut with *Pvu*II, denatured, and analyzed by electrophoresis through a 5% polyacrylamide gel. *Pvu*II cuts the DNA at a site (position 7937 [20]) 122 nucleotides downstream from the true plus-strand origin. An autoradiogram of the gel (Fig. 2) showed that in the absence of added RNA there was a low level of DNA synthesis, possibly due to the presence of contaminating priming molecules in the reverse transcriptase preparation (lane A). Specific fragments of lengths 75, 93, 190, and 430 were generated from the other *Pvu*II sites within the DNA. In the presence of RNA isolated from M-MuLV virions (Fig. 2, lane B), there was an increased amount of DNA synthesis, but the absence of fragments in the region of 122 nucleotides indicated that few, if any, initiations were occurring near the plus-strand origin. In the presence of pancreatic RNase A-treated M-MuLV RNA, however, two fragments of approximately equal intensity were produced which migrated as if they were ca. 122 nucleotides long (Fig. 2, lane C). Treatment of the reaction products with alkali before electrophoresis resulted in the loss of the slower migrating species and an increase in the faster migrating species (Fig. 2, lane D), suggesting that the slower migrating species might retain an RNA primer. Use of vector M13mp2 single-stranded DNA (containing 3 *Pvu*II sites) as the template in the reaction resulted in no fragments in the size range from 70 to 500 nucleotides (data not shown), indicating that even after RNase A treatment the priming by the M-MuLV RNA was specific to the viral sequences in the template DNA.

Analysis of the faster migrating species. The faster migrating species was eluted from a similar gel (Fig. 2, lane C), and both untreated and alkali-treated portions were submitted to

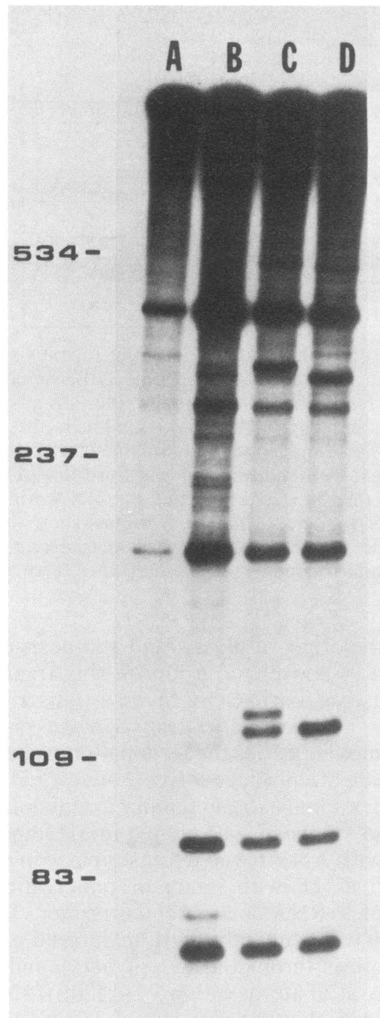

FIG. 2. Electrophoretic analysis of fragments produced by reverse transcriptase, using the M-MuLV minus-strand DNA as the template. For each lane the standard reaction (20 μl) contained 0.8 μg of the M13mp2/molA– DNA, 20 μM [α-^{32}P]dCTP (3 Ci/mmol), 4.7 U of reverse transcriptase, and either no added RNA (lane A), 0.9 μg of M-MuLV virion RNA (lane B), or 0.9 μg of virion RNA plus 1 μg of RNase A per ml (lanes C and D). After an incubation at 37°C for 60 min, the products were treated with the restriction enzyme *Pvu*II and precipitated with ethanol. The products of the reaction for lane D were, in addition, treated with 0.30 M NaOH at 60°C for 20 min before electrophoresis through a 5% polyacrylamide gel for 140 min at 7.5 V/cm. The mobilities of the SV40 *Hin*fI markers are indicated along the left side.

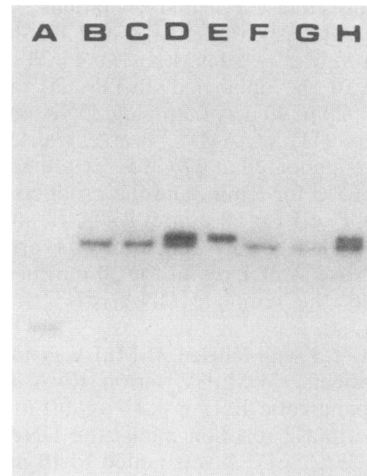

FIG. 3. Determination of the lengths of the plus-strand DNA products. Both the slower and faster migrating bands were electroeluted from a gel similar to the one shown in lane C of Fig. 2. The authentic *Pvu*II plus-strand origin fragment was similarly isolated from a reaction, using detergent-treated virions, and subsequently 5′-end labeled, using polynucleotide kinase and γ-^{32}P-labeled ATP. Lane A was the SV40 *Hin*fI marker fragment with a length of 109 nucleotides. Lane B contained the lower band which had been treated with 0.30 M NaOH for 20 min at 60°C, whereas lane C contained the untreated lower band. Lane D contained a mixture of untreated lower band material and the authentic plus-strand fragment; lane E contained only the authentic plus-strand fragment. Lanes F and G were the same as lane C. Lane H contained upper band material which had been treated with alkali. The samples were analyzed by electrophoresis for 7 h at 15 V/cm through an 8% polyacrylamide gel containing 8 M urea.

electrophoresis through an 8% polyacrylamide gel containing 8 M urea (Fig. 3, lanes B and C). The identical mobility of the fragment before and after exposure to alkali showed that this species contained no ribonucleotide residues. In principle, this fragment could be initiated at a site ~120 nucleotides to the 3′ side of any one of the three *Pvu*II sites within the M-MuLV sequences contained within the template DNA. To map the origin of the fragment, the band was eluted from a polyacrylamide gel, annealed back to the template DNA, and cut with the restriction enzyme *Sau*3AI. The presence of a single *Sau*3AI site at position 92 ± 4 within the fragment (data not shown) permits us to unambiguously assign the initiation site for this fragment to a position near the true origin for plus-strand synthesis. To determine more precisely the site of initiation of the fragment, its mobility in a sequencing gel was compared with an authentic origin fragment which had been synthesized in the endogenous reaction, cut with *Pvu*II, and end labeled, using polynucleotide kinase. Comparison of lanes C and E in Fig. 3 indicate that the faster migrating fragment primed by the RNase A-resistant oligonucleotide was one nucleotide shorter than was the fragment initiated at the correct origin. A mixture of the two samples (Fig. 3, lane D) confirms this difference. Therefore, the faster migrating species is 121 nucleotides in length.

Analysis of the slower migrating species. The slower migrating species (Fig. 2) was treated with alkali and analyzed in parallel with the previously described samples by electrophoresis through a polyacrylamide-urea gel (Fig. 3, lane H). After removal of the putative RNA primers, the DNA fragments migrated as a mixture in which about half of the strands comigrated with the 122-nucleotide-long authentic origin fragment and the other half comigrated with the fragment 121 nucleotides in length. The absence of phosphates on the 5′ ends after alkaline hydrolysis should not influence the mobility of fragments in this size range (23). These fragments originated from the same region of the template as did the faster migrating species because treatment with *Sau*3AI restriction enzyme gave the same 92-nucleotide-long fragment as that observed for the faster species. The presence of RNA on the 5′ end of the slower migrating fragment was confirmed by demonstrating transfer of ^{32}P from α-^{32}P-labeled dNTPs to ribonucleotide monophosphates after alkaline hydrolysis of the products. Although all four dNTPs were tested, transfer was only ob-

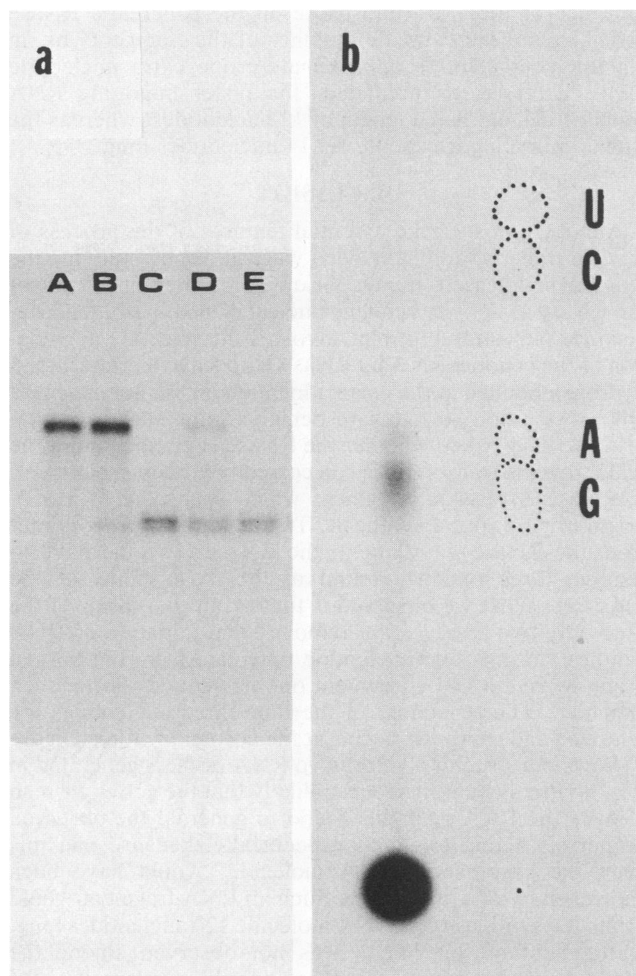

FIG. 4. Removal of RNA primers from labeled DNA fragments and analysis of DNA-RNA junctions. (Panel a) The slower migrating species was isolated from a gel similar to the one shown in lane C of Fig. 2. A portion of the fragment was analyzed directly on an 8% polyacrylamide gel containing 8 M urea (lane A) as described for Fig. 3. A second portion was treated with 0.30 M NaOH for 20 min at 60°C before electrophoretic analysis (lane C). A third portion was treated with 4.7 U of reverse transcriptase under standard reaction conditions (including dNTPs) for 60 min at 37°C (lane B). The remainder of the fragment was hybridized back to the M13mp2/molA− DNA and similarly treated with reverse transcriptase (lanes D and E). The sample in lane E was hydrolyzed in 0.30 M NaOH, as described above, before electrophoresis. All samples which had been treated with the reverse transcriptase were recut with *Pvu*II before electrophoresis. (Panel b) The slower migrating species which had been labeled with [α-^{32}P]dATP was isolated as described above. After alkaline hydrolysis the sample was analyzed by polyethyleneimine cellulose thin-layer chromatography to separate the four ribonucleotide monophosphates. The positions of the marker ribonucleotides, indicated along the right side, were located by using UV light.

served from [α-^{32}P]dATP, and in this case the only ribonucleotides labeled were AMP and GMP (Fig. 4b). These results are consistent with the above assigned lengths since a fragment 122 nucleotides long should have a primer RNA-DNA junction of riboguanylate-deoxyribosyladenine, whereas a fragment 121 nucleotides long should have a riboadenylate-deoxyribosyladenine junction (Fig. 1).

Removal of RNA primers from labeled DNA strands. The slower migrating species, containing an apparent RNA primer, was eluted from a gel similar to the one shown in Fig. 2, and the complexes were hybridized back to the template DNA. The complexes were treated with reverse transcriptase to determine whether the RNase H activity of the enzyme could remove the RNA primer (Fig. 4). Whereas incubation of the unhybridized fragments with the reverse transcriptase had no effect on their mobility in a sequencing gel (Fig. 4, lanes A and B), treatment of the hybridized fragments resulted in the removal of most of the RNA primer, yielding two DNA species with mobilities identical to the fragments produced by treatment with alkali (Fig. 4, lanes C and D). Treatment of the sample shown in Fig. 4, lane D with alkali before electrophoresis showed that the small amount of material still present as the slower migrating band after reverse transcriptase treatment was still susceptible to alkaline hydrolysis (Fig. 4, lane E). Because alkali will remove all ribonucleotides attached to the 5′ end of a DNA molecule, these results show that exposure to reverse transcriptase removed all ribonucleotides from the 5′ ends of the DNA fragments, presumably as a consequence of the RNase H activity.

RNA products resulting from RNase H removal of primers. To follow the fate of the RNA primers during the reaction with reverse transcriptase, the RNA moieties of the fragments had to be labeled. After treatment of M-MuLV RNA with pancreatic RNase, the 5′ ends were labeled with ^{32}P, using polynucleotide kinase in the presence of [γ-^{32}P]ATP. The 5′-end-labeled RNA fragments were then used to prime the synthesis of DNA strands by reverse transcriptase as described above, and the products were cut with *Pvu*II. The resulting labeled fragments were isolated from polyacrylamide gels as before and hybridized to the template DNA. These complexes were then treated with reverse transcriptase for various lengths of time, and the products were analyzed by electrophoresis through a 20% sequencing gel. If the RNA primers were removed by nibbling, then the distribution of products might be expected to have lengths ranging from 2, the minimum length observed when homopolymers were used (1), to 14, the length of the longest primer RNA. Alternatively, if the RNA primers were removed intact, the products should be a mixture of RNA molecules 13 and 14 nucleotides long (Fig. 1). Analysis of the untreated fragments (Fig. 5, lane A) consistently revealed a doublet at the position expected for the DNA fragments containing the end-labeled RNA primers. The lower band of the doublet appears to represent a mixture of labeled RNA fragments contaminating the DNA fragments and was not affected by reverse transcriptase treatment. The results of treating the RNA-primed DNA fragments for 5, 10, 20, and 60 min with reverse transcriptase (Fig. 5, lanes B through E) indicated that after 60 min essentially all of the labeled RNA was removed from the RNA-DNA complexes (upper band of the doublet) and appeared as two bands migrating at positions consistent with RNA fragment lengths of 13 and 14 nucleotides. To verify that the products had the expected lengths, DNA fragments containing the 5′-end-labeled RNA primers were subjected to mild alkaline hydrolysis, and the products were electrophoresed through a similar gel (Fig. 5, lanes G and H). The fastest migrating species of the resulting ladder was compared in other experiments to the 3′,5′-mononucleotide diphosphates and found to correspond in size to a dimer of the series. Thus, by counting up the ladder, we could determine that the slowest migrating species of the series, which had a mobility approximately equal to the faster migrating species produced by removal of the primer

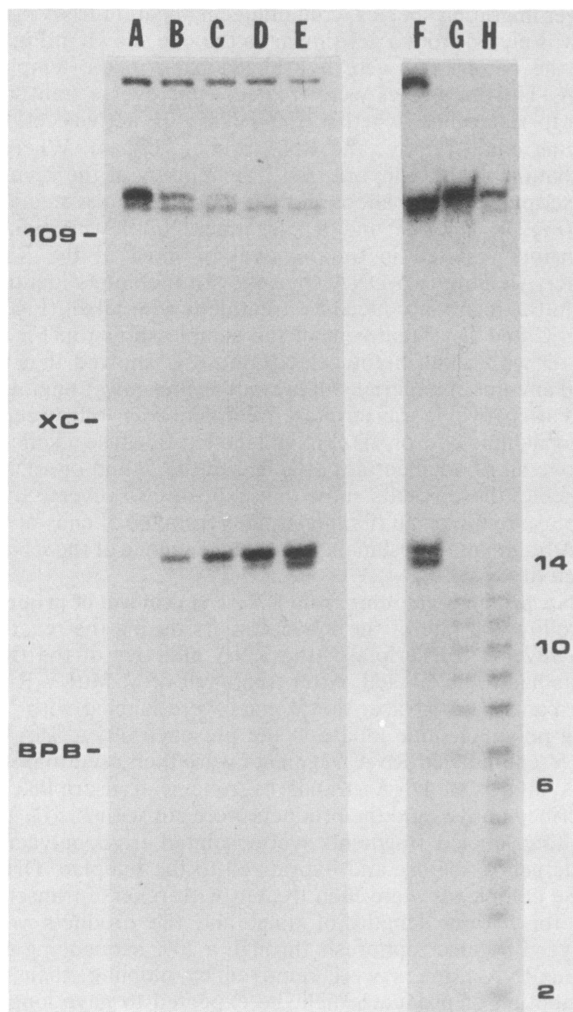

FIG. 5. Electrophoretic analysis of 5'-end-labeled RNA fragments produced by reverse transcriptase. The slower migrating band was isolated as described in the legend to Fig. 4, except that in this case the primer RNAs were labeled with ^{32}P at their 5' ends and the DNA portion of the fragment was unlabeled. The isolated fragments were hybridized back to the M13mp2/molA− DNA and incubated without reverse transcriptase (lane A) or under standard conditions in a 12-μl reaction at 37°C with 4.7 U of reverse transcriptase. Portions of 1.7 μl were removed at 5, 10, 20, and 60 min (lanes B through E, respectively) and analyzed directly by electrophoresis through a 20% polyacrylamide gel containing 8 M urea. Lane F is the same as lane E except a different preparation of the slower migrating band was used in the experiment. Lanes G and H show results of treating this second preparation of labeled fragments with 50 mM $NaHCO_3$ (pH 9.1) at 90°C for 45 and 110 min, respectively. The intermediate weaker bands seen in lane H are probably the 2',3' cyclic oligonucleotide monophosphate derivatives which are produced as intermediates under the mild alkaline hydrolysis conditions. The numbers along the right side of the figure show the lengths of several of the fragments in this series generated by the mild alkaline hydrolysis. The designations along the left side of the figure indicate the mobilities of an SV40 *Hin*fI fragment with a length of 109 nucleotides, xylene cyanol (XC), and bromophenol blue (BPB).

by the reverse transcriptase (Fig. 5, lanes F and G), had a length of 14 nucleotides. The fragments produced by the RNase H activity should end in 3'-hydroxyls, whereas pancreatic RNase leaves phosphates on the 3' ends. The presence of the extra phosphate on the pancreatic RNase series should increase the mobility of the fragments by an amount equivalent to approximately one extra nucleotide (23). Therefore, as predicted, the faster migrating RNA primer fragment had a length of 13 nucleotides, whereas the slower migrating fragment was 14 nucleotides long.

DISCUSSION

Although most of the essential features of the process of reverse transcription have been worked out by studying the reaction in detergent-treated virions, the extent of the role of the RNase H activity remains unclear. One possible mechanism for plus-strand priming involves the selective degradation of the genome RNA by RNase H to leave a primer RNA hydrogen bonded at the correct location on the minus-strand DNA. As a first step toward demonstrating such a mechanism we have asked whether the RNase H component of the AMV reverse transcriptase can correctly process a pancreatic RNase A-resistant fragment which spans the M-MuLV origin of plus-strand synthesis. To create the correct primer from the RNase A fragment, the RNase H would have to remove three residues found at the 3' terminus of the fragment. What we observed is that about two thirds of the time only two residues are removed and initiation of DNA synthesis begins one nucleotide upstream from the normal origin to give a DNA fragment one nucleotide shorter than expected. The remainder of the time three nucleotides are removed and synthesis begins at the correct location. Figure 1 shows the structures of the two RNA primers generated in this in vitro system. It seems unlikely that the activity which cleaves the RNA near the 3' end to generate the observed primers is acting totally nonspecifically, because had this been the case, some RNA molecules would have been shortened by one nucleotide. Such an RNA fragment would prime the synthesis of a DNA molecule 120 nucleotides long. A fragment of this length was not observed among the products. There are at least three possible explanations for the observed incorrect processing of the RNase A fragment. (i) Perhaps the substrate for RNase H must be longer than the 16-nucleotide RNase A fragment used in this experiment. (ii) Perhaps other factors or proteins present in the virion are required for correct cleavage of the RNA. (iii) The AMV enzyme may not be able to correctly process the M-MuLV RNA. With regard to the third possibility it is noteworthy that although the sequences surrounding the AMV and M-MuLV origins are very similar, the correct primer terminus for the AMV system is an A residue (24), whereas for M-MuLV it is a G residue. Very recently, we have shown in fact that the M-MuLV reverse transcriptase can generate the correct plus-strand primer from a DNA-RNA hybrid containing the M-MuLV plus-strand origin (W. Finston and J. Champoux, submitted for publication).

Approximately 50% of the fragments synthesized in the reactions containing the RNase A-resistant primers retained primers on their 5' ends. The basis for this observation is unknown. One possibility is that for those primers which are not removed the RNA becomes unpaired from the template and is no longer a substrate for RNase H. Such unwinding might be the result of synthesis which had initiated upstream of the origin site and extended into the region where synthesis had already occurred. Apparently no structural feature intrinsic to the fragments themselves prevents the removal of the RNA because after isolation through a polyacrylamide gel and rehybridization to the template DNA, the RNA is quite efficiently removed by reverse transcriptase.

Most noteworthy is the finding that the reverse transcrip-

tase removes the RNA primers intact. Omer and Faras (19) have similarly shown that RNase H removes the tRNA primers intact from minus DNA strands which are hybridized to plus-strand DNA. In their case, the cleavage was shown to occur at or very near the junction between the RNA and the DNA. Here we showed that the cleavage occurred precisely at the RNA-DNA junction and that no other detectable breaks were introduced into the RNA. In both studies, the requirement that the RNA moiety be in a hybrid configuration with DNA for the cleavage to occur implicates the RNase H activity of the reverse transcriptase in the reaction. There are two alternative mechanisms to account for these results. First, the RNase H may have a strong preference for RNA-DNA junctions. If so, then in addition to degrading the original template RNA, the RNase H activity is designed to specifically cut off primer RNA molecules. Second, the RNase H may instead have a preference for a minimum length of RNA-DNA duplex measured in the 5′ to 3′ direction on the RNA before it can break the RNA chain. If this length is on the order of 18 base pairs, which is the length of the tRNA primer copied by the reverse transcriptase, then on encountering a shorter RNA species the enzyme may simply cut off the longest RNA fragment possible. In the experiments reported here, this mechanism would produce the observed results in which cleavages at the RNA-DNA junctions produce RNA fragments 13 and 14 nucleotides in length. Studies with homopolymers would seem to make this second possibility unlikely. Polyadenylic acid which has been hybridized to polydeoxythymidylic acid can be degraded to over 90% solubility in trichloroacetic acid (1), which would indicate that most of the material is shorter than 10 nucleotides (3). However, more recent studies in which heteropolymeric RNA-DNA hybrids and analysis of the labeled RNA products by electrophoresis through a 20% sequencing gel were used indicates that a substantial proportion of the products (30 to 50%) have lengths in the range of 14 to 16 nucleotides (W. Finston and J. Champoux, unpublished data). Therefore, until longer RNA primers are tested in an assay similar to the one used here, one cannot unequivocally discriminate between the two mechanisms given above for RNase H removal of the RNA.

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