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Involvement of Retrovirus Reverse Transcriptase-Associated RNase H in the Initiation of Strong-Stop (+) DNA Synthesis and the Generation of the Long Terminal Repeat

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Reconstructed enzymatic reactions containing purified reverse transcriptase and defined analog substrates which mimic those purported to be natural substances for reverse transcription in vivo were employed to delineate the mechanism of strong-stop (+) DNA synthesis. Our analysis of this system has indicated that strong-stop (+) DNA synthesis is initiated after the introduction of a nick in the viral RNA genome between a polypurine sequence and an inverted repeat that represents the end of the long terminal repeat. Since inhibitors of the reverse transcriptase-associated RNase H activity prevent the introduction of the nick and the synthesis of strong-stop (+) DNA synthesis, it appears that this particular reverse transcriptase-associated enzymatic activity is responsible for the initiation of strong-stop (+) DNA. Our data also indicated that the RNase H activity creates a second nick in the viral RNA genome 11 nucleotides upstream from the strong-stop (+) DNA initiation site since the strong-stop (+) DNA synthesized in these reactions is covalently linked to an oligoribonucleotide 11 residues in length. Nucleotide sequence analysis of the oligoribonucleotide primer molecule indicated that a single homogenous oligomer was associated with strong-stop (+) DNA exhibiting the sequence rArGrGrGrArGrGrGrGrGrA. The oligoribonucleotide primer can be removed from strong-stop (+) DNA by the purified reverse transcriptase, which creates a nick at the junction between the primer and strong-stop (+) DNA. These data demonstrate that the initiation of strong-stop (+) DNA synthesis is mediated by RNase H and that the site of initiation is exactly at the end of the long terminal repeat, providing evidence for yet another function of this reverse transcriptase-associated enzymatic activity in the synthesis of retrovirus DNA.

Retroviruses replicate via a viral DNA intermediate by a complex mechanism which is becoming well understood. The linear and circular forms of viral DNA identified in virus-infected cells contain a unique structure referred to as the long terminal repeat (LTR), which provides important regulatory functions crucial to the life cycle of the virus (3, 6, 7, 12, 22, 24). These functions include initiation of transcription, termination of transcription, and integration. Although the LTR is known to be synthesized during reverse transcription of the retroviral RNA genome into the linear form of viral DNA, the exact mechanism by which it is generated has not yet been completely delineated. The generally accepted model of retrovirus DNA synthesis proposes that two transcription jumps occur during reverse transcription of the viral RNA genome to accommodate the synthesis of the LTR (1, 5, 27; for a review, see reference 28).

The first of these transcriptional jumps begins with the synthesis of strong-stop (−) DNA, which initiates on the tRNA primer molecule near the 5′ end of the viral RNA genome and terminates at the 5′ terminus of the RNA genome. After completion of strong-stop (−) DNA synthesis, the reverse transcriptase-associated RNase H activity removes the RNA portion of the RNA-DNA hybrid structure to facilitate hybrid formation between the sequences at the 3′ ends of strong-stop (−) DNA that are complementary to the viral RNA terminally redundant nucleotide sequences at the 3′ end of the same or a second viral RNA subunit (19, 26). This important reaction allows the elongation of strong-stop (−) DNA and the initiation of (+)-strand DNA synthesis to occur. The initiation of (+)-strand DNA synthesis is presumed to occur utilizing 3′ OH groups of the viral RNA genome that result from cleavage of the viral genome by an as-yet-undetermined nuclease activity (8, 25). The initial (+)-strand DNA transcript that is synthesized is unique in that it is initiated at a specific site near the 3′ end of the viral genome and terminates after transcription of the site on the viral genome to which the tRNA primer molecule binds. This species of (+)-strand DNA is referred to as strong-stop (+) DNA (29), and its synthesis is important for the second transcriptional jump which, after removal of the tRNA primer molecule from (−)-strand DNA, occurs via the complementary tRNA primer sequence present at the 3′ end of strong-stop (+) DNA and the tRNA-primer binding site sequences present at the 3′ end of (−)-strand viral DNA (5). In addition to the formation of the LTR on the linear form of viral DNA, the second transcriptional jump facilitates completion of both (+)-strand and (−)-strand DNA synthesis.

Although the site of initiation of strong-stop (+) DNA synthesis has been predicted from nucleotide sequence analysis of the LTR and strong-stop (+) DNA transcripts, the nature of the primer molecule involved in its initiation has yet to be unequivocally identified (14, 16, 17). Recently, employing RNA-DNA analog complexes that mimic the purported substrate for the initiation of strong-stop (+) DNA synthesis, we have demonstrated the presence of oligoribonucleotides at the 5′ end of strong-stop (+) DNA (20). The presence of oligoribonucleotides at the 5′ end of strong-stop (+) DNA suggests that the viral RNA genome is indeed involved as a primer in the initiation of this DNA species and that a nick is introduced in the viral RNA genome at the site of strong-stop (+) DNA to provide the reverse transcriptase with the appropriate 3′ hydroxyl for the initiation reaction to

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occur. The precise mechanism by which this nicking reaction would occur is not known, but recent studies in our laboratory have suggested that the reverse transcriptase-associated RNase H activity may play a role in this reaction by virtue of its ability to cleave RNA endonucleolytically during the removal of the tRNA primer molecule from (−)-strand DNA after completion of strong-stop (+) DNA synthesis (18).

Based upon these observations, we have pursued an analysis of strong-stop (+) DNA initiation and have obtained information that the reverse transcriptase-associated RNase H activity can indeed facilitate the initiation of strong-stop (+) DNA synthesis by the aforementioned mechanism. These data indicate that, in addition to participating in the first transcriptional jump and removal of the tRNA primer molecule from strong-stop (−) DNA, the retrovirus RNase H activity plays yet another critical role in the synthesis of retrovirus DNA and the generation of the LTRs.

MATERIALS AND METHODS

Reagents. Restriction enzymes and the Klenow fragment of DNA polymerase were obtained from New England BioLabs. *Escherichia coli* RNase H was purchased from Bethesda Research Laboratories, Inc. The αβ form of reverse transcriptase was obtained from Life Sciences, Inc., and under our conditions of enzymatic synthesis, it did not exhibit detectable nicking of viral RNA. Oligodeoxythymidylic acid-cellulose (type 7), poly(dC) · oligo$(dG)_{12-18}$, and enzymes for RNA sequencing were obtained from P-L Biochemicals, Inc. Ultrapure reagents were used for electrophoresis and density centrifugation. All other chemicals were reagent grade. [α-^{32}P]dCTP and dATP (3,000 Ci/mmol) were purchased from New England Nuclear Corp.

Isolation of poly(A)-enriched viral RNA. The Prague A and Prague C strains of avian sarcoma virus were propagated in SPAFAS COFAL/gs-negative chicken embryo fibroblasts. Virus was harvested daily and pelleted from pooled media in a type 19 rotor. The viral pellet was suspended and centrifuged through 25% sucrose in an SW27 rotor at 25,000 rpm for 2.5 h. The final viral pellet was resuspended in STE (0.1 M NaCl, 0.02 M Tris, 0.002 M EDTA [pH 7.5]) containing 0.05% sodium dodecyl sulfate and was digested with pronase at 500 μg/ml for 15 min at 37°C. After deproteination by phenol extraction, the total RNA was concentrated by ethanol precipitation. This RNA was subjected to rate zonal centrifugation in a 15 to 30% sucrose gradient in an SW41 rotor at 25,000 rpm for 7.5 h at 4°C to isolate 70S viral RNA. The 70S RNA was suspended in a low-ionic-strength buffer (0.02 M Tris, 1 mM EDTA [pH 7.4], 0.5% sodium dodecyl sulfate), denatured by heating for 5 min at 80°C, and quenched on ice; the ionic strength was raised to 0.5 M NaCl, and the RNA was applied to a 1-ml column of oligodeoxythymidylic acid-cellulose at room temperature. Non-polyadenylic acid [poly(A)]-containing RNA was washed from the support with the high salt (0.5 M NaCl) buffer, after which the poly(A)-enriched fraction was eluted with the low-ionic-strength buffer. The poly(A)-enriched RNA was put through this isolation again and was ethanol precipitated. RNA was quantitated by absorption at 260 nm with an absorption of 1 equal to 40 μg/ml.

Isolation of molecularly cloned M13 phage DNA. Molecular cloning in the single-stranded DNA bacteriophage M13mp7 was performed as described by Messing et al. (13). Recombinant phage were grown in *E. coli* JM103 and were concentrated by polyethylene glycol precipitation (31). Phage DNA was obtained by pronase treatment and phenol extraction as outlined above. The phage DNA was further treated with alkali and then was subjected to rate zonal centrifugation through a 15 to 30% sucrose density gradient in an SW41 rotor at 27,000 rpm for 16 h to eliminate any possible nonspecific primer molecules from the circular single-stranded phage DNA. DNA was quantitated by absorption at 260 nm.

Polyacrylamide gel electrophoresis. Electrophoresis through polyacrylamide gels under denaturing conditions was performed with (25 cm by 45 cm by 0.5 mm) gels containing 4 or 8% (wt/vol) polyacrylamide at an acrylamide-to-bisacrylamide ratio of 20:1 and 50% (wt/vol) urea in one-half-strength Peacock buffer as described by Sanger and Coulson (23).

Synthesis of strong-stop (+) DNA. Poly(A)-enriched RNA from 70S RNA was added to M13 single-stranded phage DNA containing a minus-sense Prague C insert complementary to a complete LTR plus *gag* sequences at a DNA-RNA ratio of 4 (wt/wt). Hybridization was performed in buffer containing either 80% formamide, 0.4 M NaCl, 0.04 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], and 0.001 M EDTA (pH 6.4) or 0.6 M NaCl, 0.02 M Tris-hydrochloride, and 0.001 M EDTA (pH 7.5) and hybridized to a C_0t of >0.01 M · s at 56 or 68°C, respectively. Identical results were obtained with both sets of hybridization conditions and with viral RNA from both strains of virus. The resulting hybrid was concentrated by ethanol precipitation and used as an analog substrate for the initiation of strong-stop (+) DNA in reconstructed reactions. The reaction conditions for synthesis consisted of 10 μg of the substrate analog per ml, 500 U of avian myeloblastosis virus (AMV) reverse transcriptase per ml, 0.1 M KCl, 0.04 M Tris-hydrochloride, 0.01 M $MgCl_2$, 1% β-mercaptoethanol (pH 8.1), 6 μM [^{32}P]dCTP (800 Ci/mmol), and 60 μM each dATP, dGTP, and TTP. After incubation for 2 h at 37°C, the reaction was treated with pronase, deproteinated by phenol extraction, and concentrated by ethanol precipitation. The total DNA product was desalted by gel filtration on Sephadex G50 to separate free isotope and was ethanol precipitated again. This material was subjected to analysis by restriction endonuclease digestion as described in the appropriate figure legends. The resulting DNA fragments were denatured in 95% formamide at 80°C for 5 min and separated by polyacrylamide gel electrophoresis under denaturing conditions. The presence of an alkali-sensitive product was examined by treating a sample with alkali in 0.3 N NaOH at 50°C for 1 h before electrophoresis.

Sequencing of the RNA primer. Alkaline-sensitive material from a restriction endonuclease digestion was visualized by autoradiography with XAR-5 film, excised from the gel, and eluted from the gel slice with 0.5 M ammonium acetate–0.01 M magnesium acetate–0.1 mM EDTA–0.1% sodium dodecyl sulfate. After recovery by ion exchange on DE-52 cellulose, samples of the material were placed in 1.5-ml Eppendorf microfuge tubes (500 cpm per tube) and were ethanol precipitated along with 3 μg of carrier yeast tRNA. The sample was suspended in 15 μl of a digestion buffer, heated at 55°C for 5 min, and then incubated at 55°C for 15 min in the presence of the appropriate nuclease. The buffers employed for the digestions were 20 mM citrate–1 mM EDTA–7 M urea (pH 5) for RNase T_1 and RNase *Phy. M.* (*Physarum polycephalum*), 20 mM citrate-1 mM EDTA–7 M urea (pH 3.5) for RNase U_2, and T_1 RNase buffer without urea for RNase *Bacillus cereus*. For partial alkaline hydrolysis of the primer, the material was suspended in 15 μl of 0.05 M $NaHCO_3$–1

mM EDTA (pH 9.0) and incubated at 90°C for 40 min after which 15 μl of 8 M urea was added (4). The samples were loaded directly onto a 8% polyacrylamide denaturing gel and electrophoresed. The material was visualized by direct radioautography on XAR-5 film.

Primer release by AMV reverse transcriptase. Samples containing an alkali-sensitive primer were isolated as described above and reannealed to M13PrC17 phage DNA to a C_0t of >0.015 M · s. Aliquot portions were alkali treated as described above, incubated with 350 U of AMV reverse transcriptase per ml or 5 U of *E. coli* RNase H per ml in the same buffer as that used in the reactions described for DNA synthesis, or incubated with the AMV reverse transcriptase in the presence of excess EDTA. The material was analyzed by electrophoresis in 8% polyacrylamide denaturing gels without deproteination.

Inhibition of the synthesis of alkaline-sensitive strong-stop (+) DNA by poly(dC) · oligo(dG)$_{12-18}$. Enzymatic reactions were performed in the presence of 5, 10, and 20 μg of poly(dC) · oligo(dG)$_{12-18}$ per ml under the reaction conditions described above but with [^{32}P]dATP as the label. The reactions were stopped in the same manner as above, and an equivalent amount of the inhibitor was added to the control before deproteinization and desalting. These samples were digested by restriction enzymes, and alkali shifts of the resultant fragments were examined by electrophoresis.

RESULTS

RNase H-mediated strong-stop (+) DNA synthesis. We have previously demonstrated the presence of oligoribonucleotides covalently associated to the 5′ end of strong-stop (+) DNA synthesized in vitro by either detergent-disrupted preparations of Rous sarcoma virus (RSV) or purified RSV RNA and reverse transcriptase (20). The fact that strong-stop (+) DNA obtained from reconstructed reactions containing purified reverse transcriptase also contains these oligoribonucleotides suggests that one of the enzymatic activities associated with the reverse transcriptase molecule participates in the introduction of a nick in the RNA genome to provide a 3′ OH primer for the initiation of strong-stop (+) DNA synthesis. Nucleotide sequence analysis of the LTRs from molecularly cloned RSV circular DNA suggests that the initiation site for strong-stop (+) DNA synthesis lies between the junction of an inverted repeat (IR_3) and a polypurine sequence of 11 nucleotides situated approximately 254 nucleotides from the 3′ end of the viral genome (9, 20, 25) (Fig. 1). However, since neither the ends of the linear forms of RSV DNA nor the oligoribonucleotide primers associated with the 5′ end of strong-stop (+) DNA have ever been sequenced, the exact nature and position of the strong-stop (+) DNA initiation site remains unknown. In an effort to shed light on this important issue, we have utilized M13 molecular cloning techniques to construct an analog of the purported natural substrate required for reverse transcriptase-mediated initiation of strong-stop (+) DNA synthesis. The constructed analog was composed of poly(A)-enriched Prague C viral RNA annealed to a (−)-strand viral DNA insert within the single-stranded DNA of the M13mp7 phage (Fig. 1). The constructed analog substrate has evident similarities to the substrate proposed to be an intermediate structure in the model of reverse transcription (5; Fig. 1). If the reverse transcriptase recognizes the analog substrate at a specific site as proposed by the model, then the initiation of strong-stop (+) DNA should occur in vitro, and newly synthesized strong-stop (+) DNA could be readily identified by the presence of the appropriate restriction endonuclease

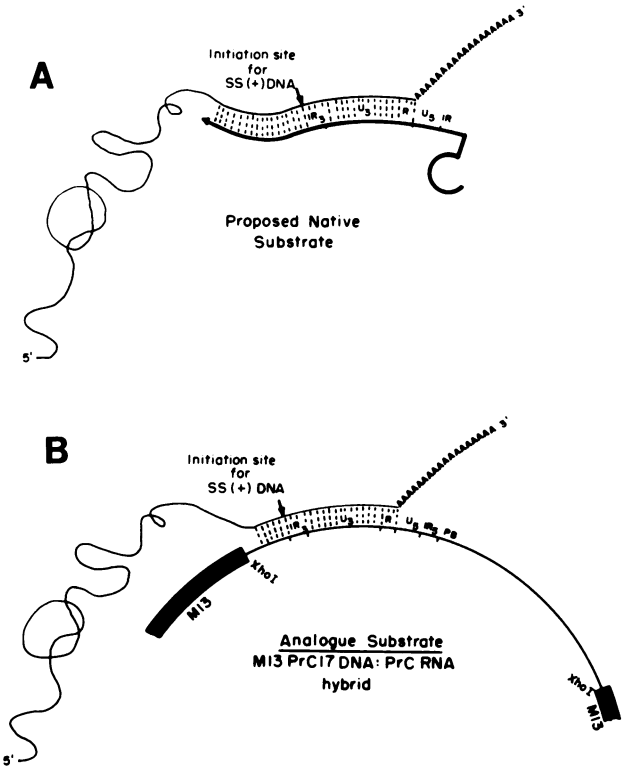

FIG. 1. Proposed model for initiation of strong-stop (+) DNA and analog substrate used to analyze initiation of strong-stop (+) DNA in reconstructed reactions. (A) Proposed native substrate, or the portion of the model for viral reverse transcription presented by Gilboa et al. (5) and Junghans et al. (8) concerning the synthesis of strong-stop (+) DNA and the proposed initiation site located internal to the 3′ end of the RNA genome between a stretch of 11 purine residues and an inverted repeat sequence. This structure results after the first transcriptional jump has occurred and before removal of the $tRNA^{trp}$ primer molecule and the second transcriptional jump, which both occur after completion of the transcription of strong-stop (+) DNA. IR_3, Inverted repeat sequence in the U_3 region constituting the 5′ end of the LTR; U_3, nucleotide sequence unique to the 3′ end of the viral RNA; R, terminal repeat of the RNA genome; U_5, nucleotide sequence unique to the 5′ end of the viral RNA; IR_5, inverted repeat sequence in the U_5 region constituting the 3′ end of the LTR; thin line, viral RNA; thick line, viral DNA; ϱ, $tRNA^{trp}$. (B) Analog substrate. An 890-base pair *Xho*I DNA fragment from Prague C avian sarcoma virus DNA containing a single LTR and molecularly cloned into plasmid pBR322 (designated pATV9 and described by Katz et al. [9]) was subcloned into a *Sal*I site of the single-stranded phage M13*mp*7. This DNA fragment consisted of a complete LTR (IR_3-U_3-R-U_5-IR_5) and the $tRNA^{trp}$ primer binding site as well as some *gag* gene sequences. A clone designated M13PrC17, containing a minus-polarity strand of the viral DNA, was identified. Poly(A) viral RNA was hybridized to this phage DNA, and the resulting complex was used as an analog substrate for the initiation of the synthesis of strong-stop (+) [SS (+)] DNA. This substrate mimics the natural substrate predicted to be involved in the initiation of strong-stop (+) DNA.

sites downstream from the initiation site (Fig. 2). Thus, the analog substrate should provide evidence that the purified reverse transcriptase can initiate strong-stop (+) DNA as well as the location of the initiation site itself.

The first indication that purified reverse transcriptase can indeed facilitate DNA synthesis from the analog substrate in reconstructed reactions containing purified reverse tran-

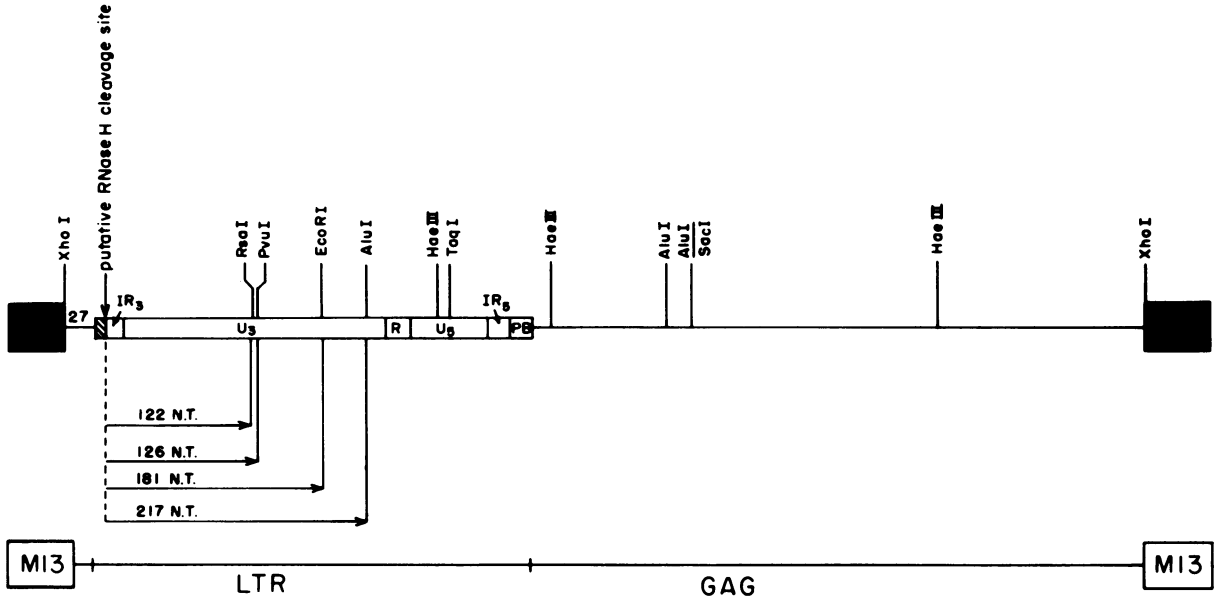

FIG. 2. Detailed restriction map of the viral DNA fragment subcloned into M13PrC17 phage. Viral DNA insert was constructed from an *Xho*I fragment generated from the plasmid pATV9 (9) and inserted into the *Sal*I site in the RF DNA of M13mp7. Restriction DNA fragments generated from the products of enzymatic reactions as outlined in the text correspond to the distances from the proposed initiation site to the specific restriction site. Restriction endonuclease sites that were mapped included *Rsa*I, *Pvu*I, *Eco*RI, and *Alu*I. Hatched box is a polypurine sequence of AGGGAGGGGGA adjacent and upstream from IR_3, which begins with the nucleotides AATGTAGTC-.

scriptase was obtained by the observed incorporation of radiolabeled deoxynucleoside triphosphates into acid-insoluble material after incubation (data not shown). (+) DNA isolated from such reactions was subjected to restriction endonuclease treatment (Fig. 3). Based upon the predicted restriction endonuclease cleavage sites for *Eco*RI, *Alu*I, and *Pvu*I, the nature of the DNA and its site of initiation were determined. (+)-strand DNA yielded specific DNA fragments of 126, 181, and 217 nucleotides when cleaved with *Pvu*I, *Eco*RI, and *Alu*I, respectively (Fig. 3A). The specificity of the DNA fragments and their nucleotide length suggests that strong-stop (+) DNA was being synthesized in these reactions and that its site of initiation was 254 nucleotides from the 3′ end of the viral genome, consistent with predictions based upon the sequence of the LTR. This specific initiation site was only observed when the analog substrate was employed as a substrate, not when either poly(A)-containing viral RNA annealed to M13mp7 DNA lacking a viral DNA insert or poly(A)-containing RNA alone was included in the reconstructed reactions.

In addition to the predicted restriction endonuclease fragments of (+) DNA synthesized in these reactions, specific DNA fragments larger by an estimated 11 nucleotides could also be detected (Fig. 3A). These larger restriction endonuclease fragments, however, were sensitive to alkali and exhibited a shift in polyacrylamide gels after alkali treatment, an observation consistent with the removal of 11 ribonucleotides from these species of strong-stop (+) DNA (Fig. 3B). By the nature of their sensitivity to alkaline hydrolysis, their resistance to physical and chemical denaturants, and their nucleotide lengths, these larger (+) DNA fragments appear to be covalently associated with the oligoribonucleotide. Further restriction endonuclease analysis of the oligoribonucleotide-containing (+) DNA fragments indicated that they contained the same restriction sites as strong-stop (+) DNA isolated from these reactions (Fig. 3A). For example, double digestion with *Pvu*I and *Eco*RI and digestion with *Pvu*I alone yielded identical (+) DNA fragments, with and without the oligoribonucleotide. The single predominant new fragment which appeared from this double digestion corresponds to the 55-nucleotide fragment between the *Pvu*I and *Eco*RI restriction sites. Since the oligoribonucleotides appear closer to the *Pvu*I restriction site than to the *Eco*RI site, they must be present on the 5′ end of the strong-stop (+) DNA synthesized in these reactions. Digestion with a variety of other restriction endonucleases substantiated these observations.

Nature of the oligoribonucleotide present at the 5′ end of strong-stop (+) DNA. In an effort to determine the exact nature of the oligoribonucleotide associated with strong-stop (+) DNA synthesized in reconstructed reactions containing the substrate analog and purified reverse transcriptase, we subjected alkaline-sensitive DNA fragments to nucleotide sequence analysis. A specific *Pvu*I DNA fragment of 137 nucleotides containing oligoribonucleotides was eluted from denaturing polyacrylamide gels and digested with base-specific RNases. The nucleotide sequence of the oligoribonucleotide primer deduced from this analysis (Fig. 4) is 5′-rArGrGrGrArGrGrGrGrGrA–strong-stop (+) DNA, consistent with the nucleotide sequence of the RNA genome present at the anticipated strong-stop (+) DNA initiation site. Several other alkaline-sensitive fragments from other restriction endonuclease digestions were also sequenced. Every alkaline-sensitive restriction endonuclease fragment sequenced yielded the same nucleotide sequence, indicating the presence of an identical oligoribonucleotide on all DNA species synthesized in these reactions (data not shown). These data indicate that the only site for (+) DNA synthesis in these reactions is at the site between the polypurine sequence and the inverted repeat IR_3.

Involvement of the reverse transcriptase-associated RNase H activity in the initiation of strong-stop (+) DNA synthesis.

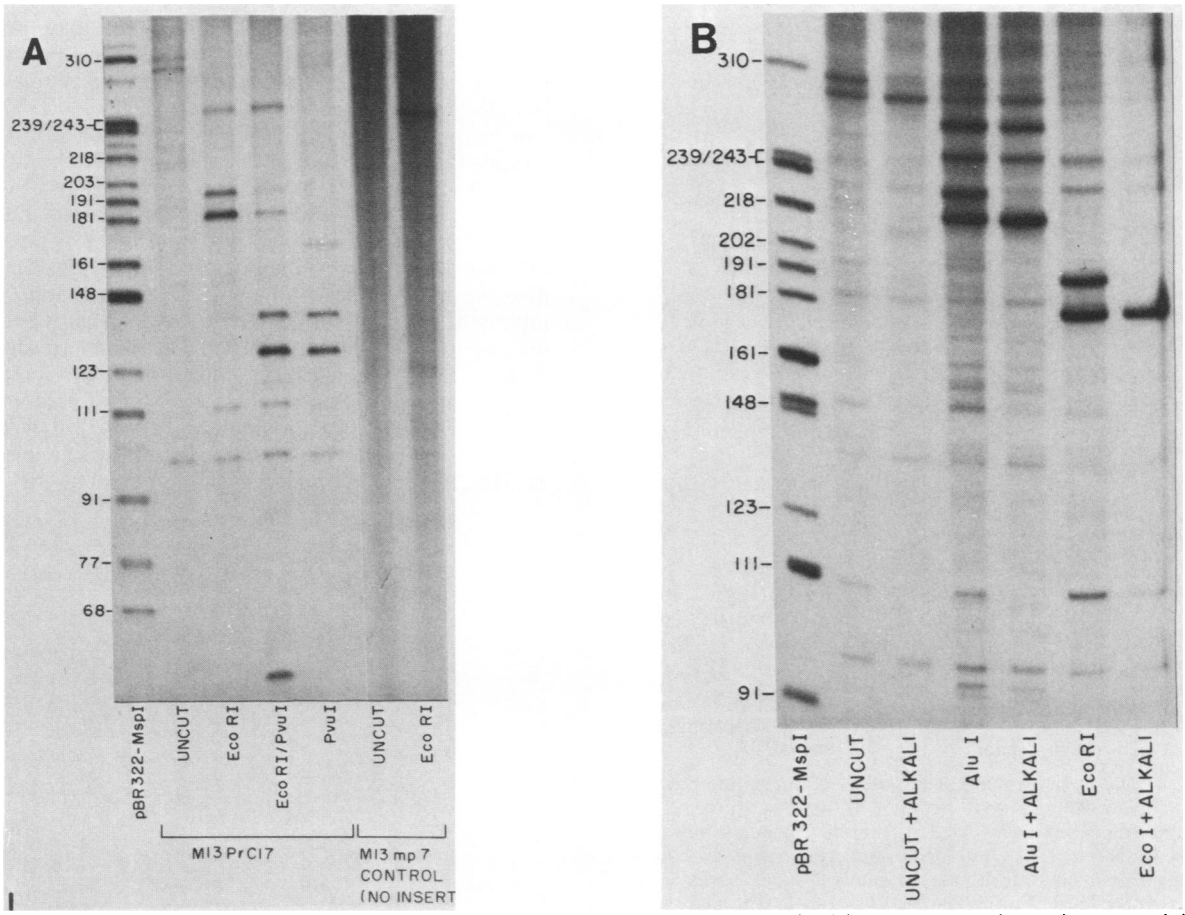

FIG. 3. Restriction endonuclease mapping and alkaline sensitivity of (+) DNA synthesized in reconstructed reactions containing the analog substrate. An enzymatic reaction containing Prague C poly(A)-containing viral RNA hybridized to either M13PrC17 or M13mp7 phage DNA in a total volume of 50 μl was incubated for 2 h as described in the text. The reaction was stopped by adding EDTA and was treated with sodium dodecyl sulfate-pronase followed by phenol extraction and gel filtration through Sephadex G-50 to remove unincorporated radioactivity. After ethanol precipitation, the DNA product was digested by the indicated restriction endonuclease in 50 μl of the required buffer. After 1 h at 37°C, 2 μg of carrier calf thymus DNA was added to the reactions, and the material was ethanol precipitated. Samples which were alkali treated were brought up in 0.3 N NaOH, incubated for 1 h at 50°C, neutralized, and ethanol precipitated. The samples were denatured in 10 μl of formamide at 80°C for 5 min and subjected to electrophoresis at 1,200 V for 3 h in a 5% polyacrylamide gel in 50% urea and one-half-strength Peacock buffer. Bands were visualized by autoradiography with XAR-5 film with an intensifying screen. (A) Demonstration of the location of restriction sites downstream from the proposed strong-stop (+) DNA initiation site. Abbreviations: pBR322-*Msp*I, *Msp*I-digested plasmid pBR322 DNA fragments labeled at their 3′ ends with ^{32}P; Uncut, total (+) DNA product synthesized in reconstructed reactions containing the analog substrate and purified reverse transcriptase not digested with restriction endonucleases; *Eco*RI, total (+) DNA product digested with *Eco*RI; *Eco*RI/*Pvu*I, total (+) DNA product digested with both *Eco*RI and *Pvu*I; *Pvu*I, total (+) DNA product digested with *Pvu*I. The last two lanes represent total radiolabeled material obtained from a reconstructed reaction containing M13mp7 without the viral DNA insert, either uncut or digested with *Eco*RI as shown. (B) Demonstration of alkaline sensitivity of the restriction DNA fragments from (+) DNA synthesized in reconstructed reaction. Abbreviations: pBR322-*Msp*I and Uncut, as in (A) above; Uncut + Alkali, total (+) DNA product treated with alkali; *Alu*I, total (+) DNA product digested with *Alu*I; *Alu*I + Alkali, total (+) DNA product digested with *Alu*I and treated with alkali; *Eco*RI, total (+) DNA product digested with *Eco*RI; *Eco*RI + Alkali, total (+) DNA product digested with *Eco*RI and treated with alkali.

We have previously demonstrated that the reverse transcriptase-associated RNase H activity was capable of removing the $tRNA^{trp}$ primer molecule from viral DNA during reverse transcription in vitro (18). One of the more interesting aspects of these studies was the demonstration that the RNase H-mediated cleavage responsible for this reaction is endonucleolytic in nature, a result extending previous notions on the possible functions of this enzymatic activity. In an effort to determine whether the reverse transcriptase-associated RNase H was responsible for the introduction of the nick into the viral RNA genome and the initiation of strong-stop (−) DNA synthesis, enzymatic reactions were performed in the presence of an inhibitor of RNase H, poly(dC) · oligo$(dG)_{12-18}$. It has been demonstrated previously that this inhibitor inhibits the reverse transcriptase-associated RNase H activity, but not its DNA polymerase or endonuclease activities (2, 15). Reconstructed reactions containing the substrate analog and purified reverse transcriptase were initiated in the presence of 10 μg of poly(dC) · oligo$(dG)_{12-18}$ and were analyzed for the presence of strong-stop (+) DNA restriction endonuclease fragments that exhibited a shift in electrophoresis in polyacrylamide gels upon alkaline hydrolysis (Fig. 5). Control reactions performed in parallel contained an equivalent amount of inhibitor added after enzymatic synthesis but before digestion with restriction endonuclease to ensure that the inhibitor did not interfere with restriction endonuclease hydrolysis. The results of these analyses indicate that the synthesis

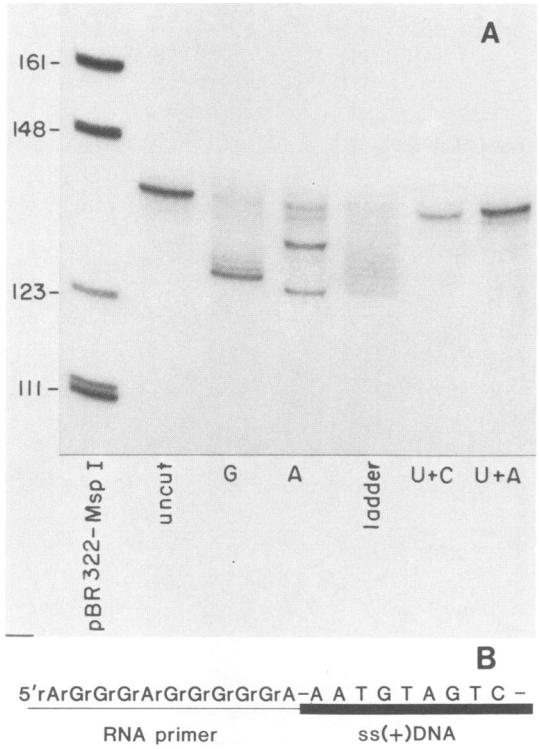

FIG. 4. Direct sequencing of the oligoribonucleotide covalently associated with strong-stop (+) DNA. (A) Alkali-sensitive (+) DNA restriction fragments were isolated from an enzymatic reaction as described in the text. The (+) DNA restriction fragments consisted of the oligoribonucleotide primer covalently attached to the 5′ end of newly synthesized [^{32}P]dCTP-labeled (+) DNA. The material was digested with 1 or 2 U of a specific RNase in 15 μl of the recommended buffer containing 3 μg of carrier yeast tRNA for 15 min at 55°C. Partial alkaline hydrolysis ladders were generated by heating the samples in bicarbonate-EDTA buffer, pH 9.0, for 40 to 60 min at 90°C in a sealed capillary tube. The samples were adjusted to 7 M urea and subjected to electrophoresis in an 8% denaturing gel (20 cm by 45 cm by 0.5 mm) without further treatment. The gel was subjected to direct autoradiography with XAR-5 film. Abbreviations: pBR322-*Msp*I, plasmid pBR322-digested with *Msp*I and end labeled with ^{32}P-unreacted, ^{32}P-labeled (+) DNA fragment not incubated with ribonucleases; uncut, see legend to Fig. 3A; G, (+) DNA treated with T_1 RNase specific for rGp-N bonds; A, (+) DNA treated with U_2 RNase specific for rAp-N bonds; ladder, partial alkaline hydrolysis ladder; U+C, (+) DNA treated with *B. cereus* RNase specific for Up-N and rCp-N bonds; U+A, (+) DNA treated with *Phy. M.* RNase specific for Up- - -N and rAp- - -N bonds. (B) Nucleotide sequence of the oligoribonucleotide primer derived from the sequencing gel shown in (A). ss(+)DNA, Strong-stop (+) DNA.

of strong-stop (+) DNA was inhibited in reactions containing poly(dC) · oligo(dG)$_{12-18}$. The observed inhibition was specific for strong-stop (+) DNA synthesis since nonspecific DNA synthesis could be observed in the presence of poly(dC) · oligo(dG)$_{12-18}$. Digestion with *Alu*I restriction endonuclease revealed the predicted alkaline-sensitive DNA fragments only in the control reactions. The synthesis of DNA in reactions containing the inhibitor apparently represents random initiation of DNA synthesis by some yet-to-be determined mechanism but serves most importantly as an internal control, indicating that poly(dC) · oligo(dG)$_{12-18}$ was not inhibiting the synthesis of strong-stop (+) DNA in these reactions by simply inhibiting DNA synthesis per se.

Release of the oligoribonucleotide primer from strong-stop (+) DNA. Ultimately, the oligoribonucleotide primer present at the 5′ end of strong-stop (+) DNA should be released from the DNA so that a complete intact linear viral DNA species can be generated. The presence of strong-stop (+) DNA species that do not contain the oligoribonucleotide primer in these reactions suggests that such a primer may be released from (+) DNA during incubation. Preliminary pulse-chase experiments containing rigorously purified reverse transcriptase and the RNase inhibitor RNasin, to ensure that any possible residual contaminating RNase activities would be inhibited, indicated that the alkaline-sensitive fragments disappeared within 1 h after chase (data not shown). To demonstrate unequivocally that the oligoribonucleotide primer could be released from strong-stop (+) DNA in these reactions, oligoribonucleotide-containing (+) DNA was isolated from reconstructed reactions and annealed to M13PrC17 single-stranded DNA containing the (−) viral

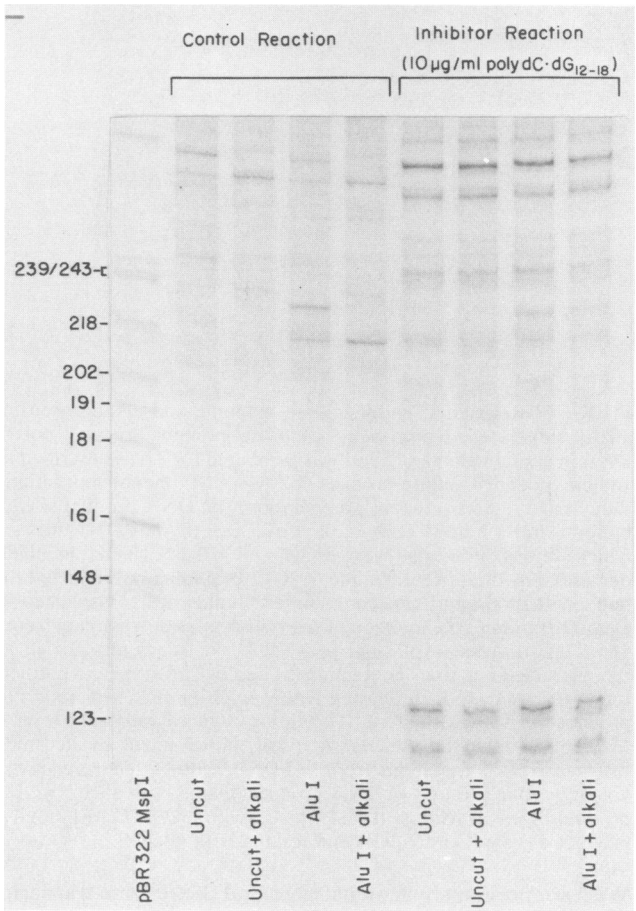

FIG. 5. Inhibition of (+) DNA synthesis by the RNase H inhibitor poly(dC) · oligo(dG)$_{12-18}$. Enzymatic synthesis of (+) DNA from the substrate analog in reconstructed reactions containing 10 μg of poly(dC) · oligo(dG)$_{12-18}$ was performed as described in the text. An equivalent amount of the inhibitor was added to control reactions after synthesis. The samples were analyzed for the presence of alkali-sensitive (+) DNA fragments upon digestion with specific restriction endonucleases. Samples were subjected to electrophoresis through a 4% polyacrylamide–50% urea gel. Abbreviations: pBR322-*Msp*I, see legend to Fig. 3A; Uncut, total (+) DNA not digested with restriction endonucleases; Uncut + alkali, total (+) DNA treated with alkali; *Alu*I, total (+) DNA digested with *Alu*I; *Alu*I + alkali total (+) DNA digested with *Alu*I and treated with alkali. Autoradiography was performed with XAR-5 film.

DNA insert. This hybrid was then employed as a substrate in reactions containing purified reverse transcriptase to determine whether this enzyme could mediate the release of the oligoribonucleotide primer from strong-stop (+) DNA. The nature of the oligoribonucleotide primer was substantiated in these studies by nucleotide sequence analysis (Fig. 6). Oligoribonucleotide primer release was assessed by the appropriate shift of the (+) DNA species in a polyacrylamide gel after incubation. Purified reverse transcriptase could indeed mediate the release of the oligoribonucleotide primer from strong-stop (+) DNA (Fig. 6). This release was directly attributable to the reverse transcriptase itself and not to general RNase contaminants present in the reaction mixtures since no shift could be observed in the presence of 20 mM EDTA, which inhibits the DNA polymerase and RNase H activities associated with reverse transcriptase but would have no effect on contaminating RNase activities. The extent of the shift reveals that the primer is released by cleavage at the junction between the last ribonucleotide of the primer and the first incorporated deoxyribonucleotide. Digestion with *E. coli* RNase H indicates that the substrate exists in a hybrid configuration. Interestingly, the *E. coli* RNase H activity cannot cleave the hybrid at the junction between a ribonucleotide and a deoxyribonucleotide, substantiating similar findings on the RNase H-mediated removal of $tRNA^{trp}$ from (−) DNA (18). Release of the oligoribonucleotide primer did not occur if the hybrid was denatured before incubation with reverse transcriptase.

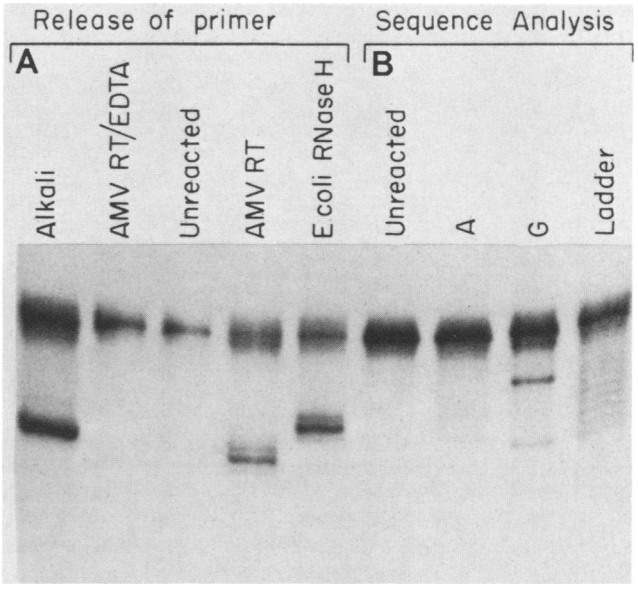

FIG. 6. Release of the oligoribonucleotide primer from (+) DNA during incubation with purified reverse transcriptase. An alkali-sensitive (+) DNA fragment was isolated as described in the text and was hybridized to 2 μg of M13PrC17 to a C_0t of >0.015 M · s. A portion of the same (+) DNA fragment was used for sequencing as previously described. Reactions for primer release contained 2,000 dpm of the substrate incubated with no enzyme, with 350 U of AMV reverse transcriptase per ml plus 20 mM EDTA, with 350 U of AMV reverse transcriptase per ml, or with 5 U of *E. coli* RNase H per ml for 2 h at 37°C in the same buffer conditions used for DNA synthesis. Carrier DNA was added, and the samples were recovered by enthanol precipitation. The samples were denatured in formamide and analyzed by electrophoresis in denaturing 8% polyacrylamide gels. (A) Abbreviations: Alkali, substrate treated with alkali for 1 h; AMV RT/EDTA, substrate incubated with reverse transcriptase and EDTA; Unreacted, substrate incubated in buffer without reverse transcriptase; AMV RT, substrate incubated with reverse transcriptase; *E. coli* RNase H, substrate incubated with *E. coli* RNase H. (B) Abbreviations: Unreacted, sample incubated without enzyme; G, sample treated with RNase T_1; A, sample treated with U_2 RNase; Ladder, samples treated under condition of partial alkaline hydrolysis to form ladder.

DISCUSSION

Our data indicate that when provided with the appropriate substrate, the avian retrovirus reverse transcriptase-associated RNase H creates a specific nick in the viral RNA genome to provide a 3′ OH for the initiation of strong-stop (+) DNA synthesis to occur. Although nucleotide sequence analysis of the LTR obtained from molecularly cloned circular viral DNA from infected cells predicted the location of the initiation site for strong-stop (+) DNA synthesis, unequivocal proof of this conjecture was not attainable with this information. Both restriction endonuclease analysis of the strong-stop (+) DNA synthesized in these reactions and the nucleotide sequence of the oligoribonucleotide covalently associated with strong-stop (+) DNA positioned the initiation site unequivocally between the polypurine sequence and the inverted repeat nucleotides of the 3′ end of the viral RNA genome (Fig. 7).

The studies reported here further extend the role of the reverse transcriptase-associated RNase H activity in the synthesis of retrovirus DNA intermediates. The fact that this activity was capable of creating an endonucleolytic nick at the junction between $tRNA^{trp}$ and (−) DNA to remove the (−) DNA primer molecule suggested its possible participation in strong-stop (+) DNA synthesis (18). In contrast to the previous studies based upon reactions containing synthetic homopolymeric substrates, which defined the retrovirus RNase H activity as a processive exonuclease (10, 11), studies with defined analog substrates of purported natural substrates have indicated the true nature of this activity and stress the need for employing such substrates to elucidate the full capabilities and involvement of other reverse transcriptase-associated enzymatic activities in the synthesis and integration of viral DNA.

Based upon the studies reported in this communication, it appears that the RNase H activity can create a second nick approximately 11 nucleotides upstream from the strong-stop (+) DNA initiation site, thereby generating an oligoribonucleotide primer 11 nucleotides in length at the 5′ end of strong-stop (+) DNA (Fig. 7). As enzymatic synthesis continues, the oligoribonucleotide primer is released from strong-stop (+) DNA by yet another RNase H-mediated cleavage at the primer (+) DNA junction. Thus, the retrovirus RNase H activity can introduce a nick to initiate DNA synthesis as well as cleave the RNA primer molecules from both (−) and (+) DNA species.

The specificity of these cleavage reactions reveals information concerning nucleotide sequence recognition sites as well as enzyme binding sites. For instance, the specificity of the cleavage reaction appears to be directed by a specific nucleotide sequence immediately adjacent to the cleavage site. In the removal of $tRNA^{trp}$ from (−) DNA, a nucleotide sequence, AATGAAGCCTTCTGC, resembling the inverted repeat of the U_3 region, is present adjacent to the cleavage site. The sequence exhibits a 12-of-15-nucleotide homology with the sequence AATGTAGTCTTATGC immediately adjacent to the site in which strong-stop (+) DNA initiates. Thus, this particular nucleotide sequence appears to contribute to the specificity of the cleavage reaction.

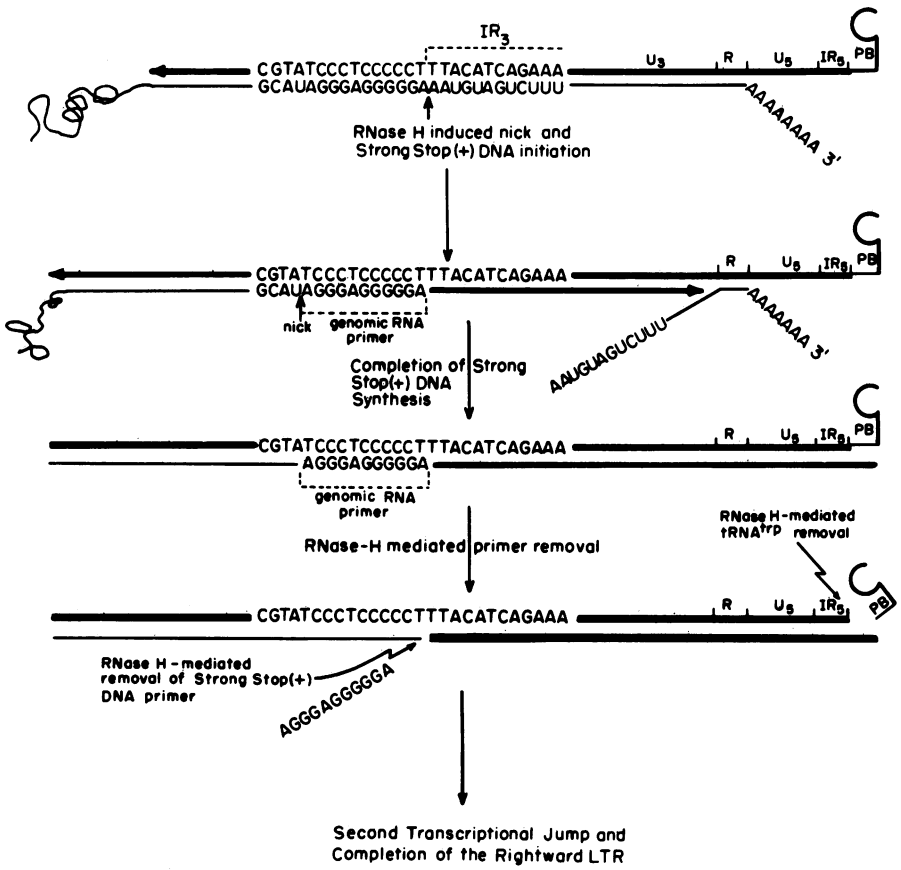

FIG. 7. Proposed model of RNase H-mediated strong-stop (+) DNA initiation and release of genomic RNA primer. Symbols: thin line, viral RNA; thick line, viral DNA; ⌒, $tRNA^{trp}$ primer; R, genomic terminal redundancy; U_3, nucleotide sequences unique to the 3′ end of the viral genome; U_5, nucleotide sequences unique to the 5′ end of the viral genome; PB, primer binding site.

The second important parameter for RNase H-mediated cleavage appears to be the presence of specific sequences or secondary structural features for binding of the reverse transcriptase at or near the site of cleavage. In the removal of $tRNA^{trp}$ from (−) DNA, the important feature for binding might be the $tRNA^{trp}$ primer molecule itself since previous studies have directly demonstrated that the reverse transcriptase molecule can bind to the $tRNA^{trp}$ primer molecule (21). In the RNase H-mediated cleavage of the viral RNA genome before the initiation of strong-stop (+) DNA, the important feature required for binding might be the polypurine sequence adjacent to the cleavage site since it is also known that polypurine sequences can bind to reverse transcriptase (30). In fact, the inhibition of RNase H with poly(C) · oligo$(dG)_{12-18}$ may simply represent specific binding of this inhibitor to the reverse transcriptase, resulting in an inhibition of activity by competition. Thus, two specific events appear to be required to confer specificity of retrovirus RNase H-mediated cleavage: first, binding of the enzyme to regions of the substrate that contain sequences or structures that facilitate binding of the reverse transcriptase molecule; and second, an inverted repeated structure with a sequence at least 80% homologous to IR_3. It will be of considerable interest to prove this contention directly by determining whether other regions of the RSV genome contain nucleotide sequences consisting of a polypurine stretch adjacent to an inverted repeat-like sequence and whether defined hybrid structures of these regions of the RSV genome represent appropriate sites for cleavage.

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