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Evidence for Involvement of an RNA Primer in Initiation of Strong-Stop Plus DNA Synthesis During Reverse Transcription In Vitro

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Employing enzymatic reactions in vitro, we have identified the presence of oligoribonucleotides at the 5′ end of strong-stop plus [(+)] DNA. Similar results were obtained whether the strong-stop (+) DNA was synthesized by preparations of detergent-disrupted avian sarcoma virus or reconstructed reactions containing purified reverse transcriptase and a template that mimics the purported natural template for strong-stop (+) DNA synthesis. The latter reactions provide a system to delineate more precisely the discrete requirements necessary for the initiation and synthesis of this species of (+) DNA.

During the course of retroviral replication, a linear double-stranded DNA molecule is synthesized from the single-stranded viral genomic RNA in the cytoplasm of infected cells (30). One of the more interesting structural features of the resultant linear double-stranded DNA molecule is the presence of large terminal repeats (LTRs) which are composed of sequences derived from both the 5′ end and the 3′end of viral RNA (7, 9, 22). A model based upon several features of both the retrovirus RNA genome and associated reverse transcriptase activities has been proposed to explain the mechanism by which the LTRs are generated during DNA synthesis (7). One of the major features of this model involves the location of the initiation points of minus [(−)] DNA synthesis (DNA complementary to viral RNA) and plus [(+)] DNA synthesis (DNA of the same polarity as viral RNA) and their relationship to the ends of the LTRs.

The mechanism of (−) DNA initiation has been well documented and involves the use of a tRNA primer hydrogen bonded near the 5′ end of the template viral RNA (23, 26, 28, 29). This tRNA ($tRNA^{trp}$ for avian retroviruses, $tRNA^{pro}$ for murine retroviruses [30]) provides the 3′ OH from which the synthesis of (−) DNA begins. The first (−) DNA species termed strong-stop (−) DNA contains sequences from the 3′ OH of the tRNA primer molecule through to the 5′ end of the viral RNA (29).

The mechanism of (+) DNA initiation is less well understood. It appears that the first species made during viral DNA synthesis contains sequences representing both the 5′ end and the 3′ end of the viral genome (15, 16, 29). This DNA, known as strong-stop (+) DNA, is initiated near the 3′ end of the genome and copies 3′ and 5′ genomic sequences from (−) DNA. Strong-stop (+) DNA appears to terminate after copying the region of the $tRNA^{trp}$ primer molecule that binds to the viral RNA genome (30). The initiation point for strong-stop (+) DNA has been shown in murine leukemia virus to be precisely at the 3′ end of the LTR as predicted by the model of reverse transcription described by Gilboa et al. (7).

Although several investigators have attempted to identify a primer for strong-stop (+) DNA initiation, none has been successful to date. Employing reconstructed reactions containing purified reverse transcriptase and viral RNA, several groups did indeed demonstrate that (+) polarity DNA synthesized in these reactions did contain covalently attached RNA (17, 18). These data, however, did not discern whether or not the (+) DNA made was strong-stop (+) DNA and were therefore subject to the criticism that the initiation points observed were spurious, resulting from low levels of nicking of the viral genome by contaminating RNases known to be present in partially purified preparations of reverse transcriptase.

In an effort to understand the mechanism of strong-stop (+) DNA initiation, we have examined strong-stop (+) DNA species synthesized in enzymatic reactions in vitro containing detergent-disrupted preparations of avian sarcoma virus (ASV). Our data indicate that the strong-stop (+) DNA species synthesized in these reactions contain 3 to 10 alkali-sensitive nucleotides at their 5′ ends. We have also shown that similar species of (+) DNA containing covalently attached oligoribonucleotides can be synthesized in vitro with only viral RNA hybridized to (−) DNA used as a template for the purified reverse transcriptase. This latter system should allow us to define more precisely the mechanism by which strong-stop (+) DNA is initiated by oligoribonucleotides during reverse transcription in vitro.

MATERIALS AND METHODS

Virus and viral RNA. The Prague A and Prague C strains of ASV were propagated in chicken embryo fibroblasts prepared from 10-day-old gs^- chf^-, c/o embryonated chicken eggs purchased from SPAFAS (Roanoke, Ill.). Virus from tissue culture media of infected fibroblasts was concentrated as described previously (2, 5) and used for detergent-disrupted DNA synthesis reactions or for the isolation of viral RNA.

Viral RNA was isolated from concentrated virus by sodium dodecyl sulfate (SDS, 0.5%)–pronase (500 μg/ml) treatment at 37°C for 30 min and subsequent phenol extraction and ethanol precipitation. This RNA was fractionated into 70S and 4/7S RNA by using sucrose density gradients (2, 5). The 70S RNA was then heat denatured, and polyadenylic acid-containing viral RNA was isolated by oligodeoxythymidylic acid-cellulose chromatography (1).

In vitro DNA synthesis. DNA synthesis by detergent-disrupted virus was accomplished by a method described previously for the preparation of strong-stop (+) DNA, except that the concentration of the labeled nucleotide was raised to 160 μM and no actinomycin D was added (19).

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ligase, and large fragments of *Escherichia coli* DNA polymerase I were purchased from New England BioLabs, Beverly, Mass., and Bethesda Research Laboratories, Gaithersburg, Md. Reverse transcriptase was obtained from Life Sciences, Inc., St. Petersburg, Fla. α-^{32}P-labeled deoxynucleotide triphosphates were purchased at 800 Ci/mmol from Amersham Corp., Arlington Heights, Ill., and at 3,000 Ci/mmol from New England Nuclear Corp., Boston, Mass.

Molecular cloning in bacteriophage M13 and M13 DNA isolation. Cloning in the single-stranded phage M13 mp7 was as described (14). Phage were grown in *E. coli* JM103 and were precipitated with polyethylene glycol (31). Phage DNA was then obtained by phenol extraction and ethanol precipitation.

Polyacrylamide gel electrophoresis. Electrophoresis of nucleic acids was performed in 5 or 8% polyacrylamide (20:1 acrylamide bisacrylamide)–8 M urea gels (45 cm by 25 cm by 0.5 mm) in 90 mM Tris-borate–1 mM EDTA, pH 8.3, as described previously (21).

^{32}P-labeled DNA markers. DNA markers were prepared by restriction cleavage of plasmid pBR322 with either *Hin*fI or *Msp*I (24). The restriction fragments were labeled at their 3′ ends with the large fragment of *E. coli* DNA polymerase I and an α-^{32}P-labeled deoxynucleotide triphosphate (dATP for *Hin*fI, dCTP for *Msp*I) (12).

Purification of strong-stop (+) DNA. Strong-stop (+) DNA purified from reactions containing detergent-disrupted preparations of virus was in the following manner. Total ^{32}P-labeled DNA from reactions containing detergent-disrupted virus was electrophoresed in 5% polyacrylamide–8 M urea gels as described. DNA of approximately the size expected for strong-stop (+) DNA (330 to 350 nucleotides) was eluted from the gel slice (13) and ethanol precipitated. This DNA was hybridized to 5 μg of single-stranded M13 phage minus-strand DNA containing an *Eco*RI fragment obtained from molecularly cloned Prague A ASV DNA (M13 · PrA2) (8). The *Eco*RI fragment contained a permuted LTR region (11). The hybridization was performed to a C_0t value of 1.0 at 68°C in 0.6 M NaCl–20 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA. The hybrids were then sedimented through a 15 to 30% sucrose gradient in a Beckman SW60 rotor at 60,000 rpm for 210 min and were fractionated. Strong-stop (+) DNA hybridized to M13 PrA2 sedimented in the bottom 25% of the gradient, and unhybridized material remained at the top 25% of the gradient.

Strong-stop (+) DNA was synthesized and purified from reconstructed reactions as follows. Strong-stop (+) DNA was synthesized by using polyadenylic acid-containing Prague C ASV RNA annealed to a single-stranded M13 phage DNA which contained a (−) sense *Xho*I restriction fragment with a permuted LTR sequence isolated from molecularly cloned Prague C DNA as a template primer for purified avian myeloblastosis virus reverse transcriptase. The hybrid was incubated to C_0t = 0.06 M · s in 80% formamide–0.4 M NaCl–0.04 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)]–0.001 M EDTA (pH, 6.8) at 56°C (3) with a DNA-to-RNA ratio of 4 (wt/wt). The phage DNA for the template was isolated as described above and treated with 0.3 M NaOH at 50°C for 1 h followed by rate-zonal centrifugation through a 15 to 30% sucrose gradient to eliminate the possibility of spurious priming from any nonspecific low-molecular-weight fragments that might have been present.

A typical reconstructed reaction contained 50 μg of template primer per ml, 525 U of avian myeloblastosis virus reverse transcriptase per ml, and 48 μM dATP, dGTP, and dTTP plus 75 μCi of [^{32}P]dCTP in 0.1 M KCl–0.01 M $MgCl_2$–1% β-mercaptoethanol. After 1.5 h at 37°C, cold dCTP (5 μM) was added, and the reaction was incubated for another 1.5 h. After pronase treatment, deproteination by phenol extraction, and several ethanol precipitations from 2.5 M ammonium acetate, the product was analyzed by restriction endonuclease digestion. Half of each digest was treated with alkali and precipitated with ethanol again before separation by polyacrylamide gel electrophoresis.

RESULTS

Kinetics of strong-stop (+) DNA synthesis. The kinetics of DNA synthesis by detergent-disrupted preparations of ASV under reaction conditions that facilitate the synthesis of strong-stop (+) DNA is presented in Fig. 1. After 30 min of enzymatic synthesis, multiple discrete species of DNA, including strong-stop (−) DNA and strong-stop (+) DNA, could be readily detected. Strong-stop (−) DNA decreased in relative intensity with a corresponding increase in longer DNA transcripts as enzymatic synthesis proceeded, reflecting its elongation on the 3′ end of the viral RNA (20). In contrast, the amount of strong-stop (+) DNA present in these reactions stayed relatively constant with time since this species of DNA can only be elongated after (−) DNA synthesis has terminated after transcription of the tRNA primer binding site (6), a reaction that does not proceed efficiently under these conditions of enzymatic synthesis. The identity of strong-stop (+) DNA was confirmed by its absence in reactions performed in the presence of actinomycin D (data not shown) and by its isolation in the purification scheme outlined above and subsequent restriction enzyme analysis described in detail in the following section.

Termination of strong-stop (+) DNA. Strong-stop (+) DNA synthesized by detergent-disrupted ASV as described above was isolated and analyzed by electrophoresis on polyacrylamide gels after digestion by restriction endonucleases and alkaline hydrolysis. Restriction endonuclease analysis of strong-stop (+) DNA with *Hin*fI, *Alu*I, and *Eco*RI revealed the presence of two 5′-terminal fragments differing in length by 10 nucleotides (Fig. 2 and 3, Table 1) suggesting the presence of two initiation points. This finding most likely reflects either heterogeneity in the LTR of the Prague A virus stocks used in these studies or the presence of contaminating Rous-associated virus in the Prague A virus stocks (10, 25). The additional 10 nucleotides present on the larger of the two 5′-terminal restriction endonuclease fragments lie between the *Eco*RI site and the *Rsa*I site (Fig. 3) since cleavage of the strong-stop (+) DNA by *Rsa*I yielded a single 5′-terminal fragment. Based on this restriction endonuclease analysis and DNA sequencing data (10, 25), it appears that this slightly smaller species of strong-stop (+) DNA corresponds to the Prague A strain of ASV and the slightly longer (10 nucleotides) species corresponds to a variant that may have spontaneously arisen during passage of ASV or the presence of Rous-associated virus-like virus (10). With this information considered, the site of initiation of strong-stop (+) DNA synthesized in vitro can be positioned 125 nucleotides from the *Rsa*I site. Thus, strong-stop (+) DNA is initiated at the end of the LTR sequence, as demonstrated previously for murine leukemia virus (15).

The 3′ termini of the strong-stop (+) DNA species are located in three clusters: (i) immediately adjacent to the $tRNA^{trp}$ primer molecule, (ii) 6 to 8 nucleotides from the 3′ end of $tRNA^{trp}$, and (iii) 17 nucleotides from the 3′ end of the $tRNA^{trp}$ primer (Fig. 2–4). These data suggest that several species of strong-stop (+) DNA which contain transcripts

that copy only part of the $tRNA^{trp}$ primer molecule can be identified in enzymatic reactions in vitro.

Alkali sensitivity of nucleotides at the 5′ end of strong-stop (+) DNA. The sensitivity of strong-stop (+) DNA to alkaline hydrolysis was assessed by comparing alkali-treated and untreated samples after restriction endonuclease hydrolysis (Fig. 4). Such a comparison revealed that the 5′-end restriction endonuclease fragments of strong-stop (+) DNA consisted of several discrete species containing 3 to 10 ribonucleotides that were removed by alkali treatment. For example, by employing the restriction endonuclease *Rsa*I, a second alkali-sensitive band, approximately 3 nucleotides longer than the 5′-end fragment, could be observed before alkaline hydrolysis (Fig. 2, 4). After *Eco*RI digestion, the larger bands from the untreated samples appeared to be about 10 nucleotides longer than the alkali-treated 5′-end fragments of strong-stop DNA. These data indicate the presence of oligoribonucleotides at the 5′-end of strong-stop (+) DNA species synthesized in vitro.

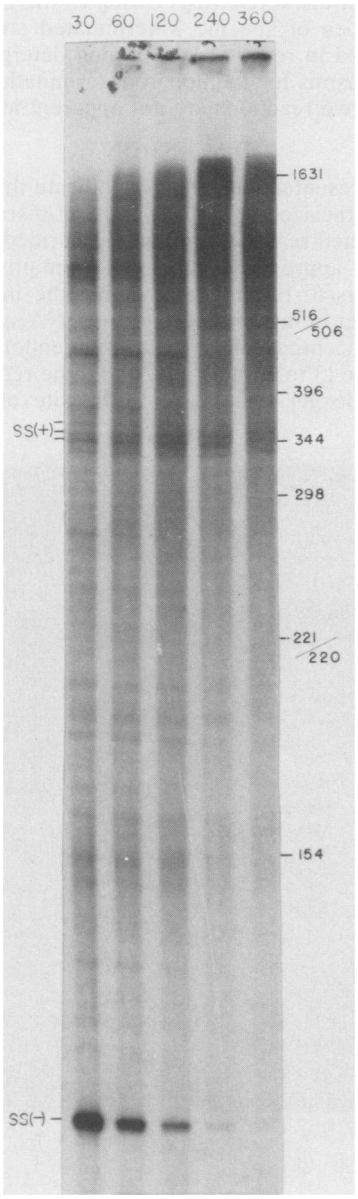

FIG. 1. Kinetics of DNA synthesis by detergent-disrupted ASV reaction. Enzymatic synthesis of DNA employing detergent-disrupted virus was performed as described in the text with [^{125}I]dCTP as the labeled nucleotide (4). Samples (100 μl) were removed at 30, 60, 120, 240, and 360 min, and the total nucleic acid product from each was RNase treated, phenol extracted, and ethanol precipitated. DNA samples from each time point (10,000 cpm) were then electrophoresed in a 5% acrylamide–8 M urea gel as described in the text. The lanes are labeled with the time point (in minutes) of the samples they contain. The positions of 3′-^{32}P-labeled, *Hin*fI-cleaved, pBR322 strong-stop (+) DNA [SS(+)] and strong-stop (−) DNA [SS(−)] are indicated.

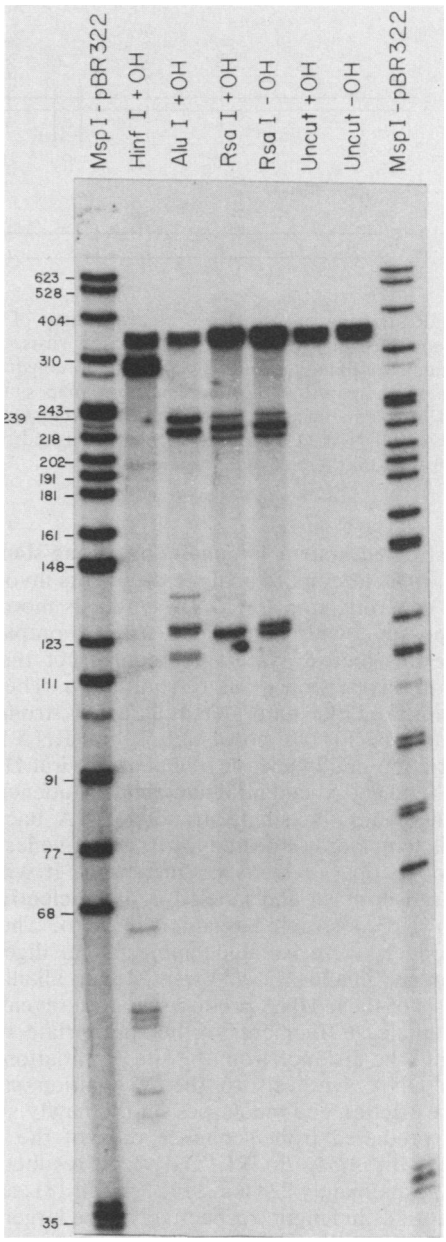

FIG. 2. Restriction endonuclease analysis of purified strong-stop (+) DNA from detergent-disrupted virus DNA synthesis reactions. ^{32}P-labeled strong-stop (+) DNA isolated as described in the text was digested with the indicated restriction endonucleases and electrophoresed in an 8% polyacrylamide–8 M urea gel as described in the text. The lengths of 3′-^{32}P-labeled, *Msp*I-cleaved pBR322 DNA are indicated at the left.

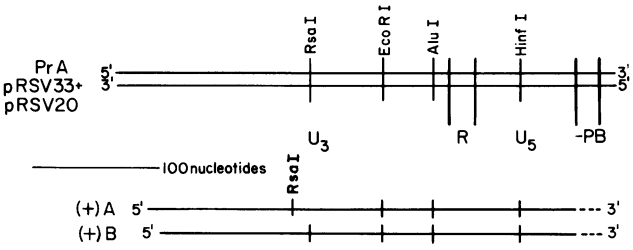

FIG. 3. Restriction endonuclease cleavage map of strong-stop (+) DNA synthesized from detergent-disrupted virus. The restriction endonuclease data from Fig. 2 were used to construct the map shown. It is compared with the restriction map obtained from previous DNA sequencing data obtained from molecularly cloned Prague A ASV DNAs (11; C. A. Omer, unpublished results). See Table 1 for data on the cleavage fragments.

Reconstructed system for analyzing strong-stop (+) DNA initiation. In an attempt to analyze the events involved in the initiation of strong-stop (+) DNA synthesis more precisely than is possible with detergent-disrupted preparations of ASV, we designed a system to reconstruct the template involved in strong-stop (+) DNA initiation. The template, which mimicked the natural template for strong-stop (+) DNA synthesis (Fig. 5), consisted of viral RNA hybridized to a restriction endonuclease fragment of viral DNA representing 3'-end and 5'-end viral nucleotide sequences molecularly cloned into the single-stranded DNA bacteriophage M13. This template was tested for its effectiveness at generating strong-stop (+) DNA by incubating it with purified reverse transcriptase and labeled deoxynucleotide triphosphates in a reconstructed reaction in vitro. The resultant DNA products were isolated, subjected to digestion with restriction endonucleases, and treated with alkali. Gel electrophoresis of these DNA products (Fig. 6) revealed restriction endonuclease fragments of the appropriate sizes corresponding to the distance from the site of initiation of strong-stop (+) DNA synthesis to the recognition site for the specific restriction endonucleases on the newly synthesized DNA as predicted from sequence data of the LTR (11). Cleavage with *Alu*I, *Eco*RI, and *Pvu*I resulted in DNA species approximately 228 and 217, 192 and 181, and 136 and 126 nucleotides in length, respectively. The larger species in each pair showed alkali lability and therefore represented the 5' termini of strong-stop (+) DNA. *Rsa*I cleavage also revealed two major DNA species of 125 and 122 nucleotides in length, again with the larger species exhibiting alkali sensitivity. The presence of fewer oligoribonucleotides on the *Rsa*I fragment presumably reflected hydrolysis by a RNase activity present in commercially prepared *Rsa*I (data not shown). No similar specific DNA bands were seen with the use either of M13 phage DNA lacking the ASV DNA fragment or of viral RNA alone (data not shown). Similarly, no DNA bands were observed if the M13-ASV DNA recombinant and unhybridized viral RNA were present in the enzymatic reactions. *Eco*RI hydrolysis revealed patterns similar to those obtained for the strong-stop (+) DNA products isolated from reactions containing detergent-disrupted virus (Fig. 2 and 4). However, the electrophoretic profiles were less complex than those of the DNA product from detergent-disrupted virus owing to the absence of the multiple species of specific 3'-terminated strong-stop (+) DNA observed in reactions containing detergent-disrupted virus. The reasons for the apparent termination differences between the two reactions are not apparent at this time.

TABLE 1. Restriction endonuclease cleavage fragments of strong-stop (+) DNA synthesized from detergent-disrupted virus[a]

Restriction endonuclease	Cleavage sites (nucleotides)	
	5' terminal	3' terminal
*Hin*fI	305 (A), 295 (B)	65, 54, 45
*Alu*I	234 (A), 224 (B)	138, 125, 118
*Eco*RI	191 (A), 181 (B)	179, 159, 151
*Rsa*I	125 (B)	237, 230, 218

[a] See Fig. 3, for map.

DISCUSSION

The data presented in this paper indicate that strong-stop (+) DNA synthesized either by detergent-disrupted virus or by reconstructed reactions containing purified reverse transcriptase and appropriate substrates contains oligoribonucleotides at its 5' termini, implicating the involvement of RNA as primer in the initiation of this DNA species. Within the limits of accuracy of the restriction endonuclease mapping technique (3 to 5 nucleotides) and the resolution of the polyacrylamide gel system used, the site of initiation of

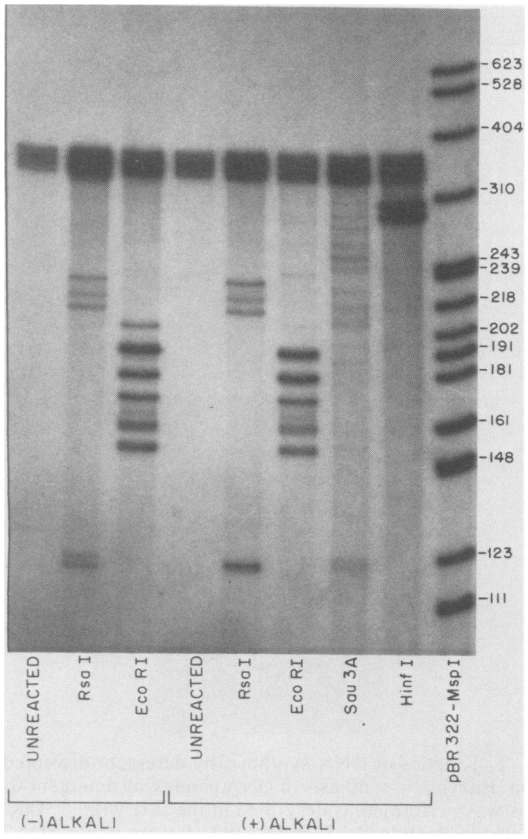

FIG. 4. Alkali sensitivity of strong-stop (+) DNA synthesized from detergent-disrupted virus. Strong-stop (+) DNA was isolated from enzymatic reactions containing detergent-disrupted virus as described. Samples of strong-stop (+) DNA were divided into two portions, one of which was treated with alkali after restriction endonuclease treatment and was electrophoresed in polyacrylamide gels as described in the text. The last lane contained the pBR322 *Msp*I nucleotide length markers.

strong-stop (+) DNA synthesized in these reactions in vitro corresponds to the 5′ end of the LTR sequence. This is in agreement with the data from murine leukemia virus (15).

The 3′ ends of strong-stop (+) DNA as determined by restriction analysis occur in three clusters (Fig. 2–4). One is at the 3′ end of the $tRNA^{trp}$ (−) DNA primer, another is 6 to 8 nucleotides into $tRNA^{trp}$, and a third is 17 nucleotides into $tRNA^{trp}$. These correspond to the putative termini determined by Taylor and Hsu (27) and Gilboa (7). The copying of the 3′ end of $tRNA^{trp}$ is required for its removal from (−) DNA by the ribonuclease H activity of reverse transcriptase (19).

The oligoribonucleotides associated with strong-stop (+) DNA synthesized by detergent-disrupted virus ranged from 3 to 10 nucleotides in length. These oligoribonucleotides were present on some, but not all, of the strong-stop (+) DNA isolated from these reactions, suggesting that removal of the oligoribonucleotides occurs from strong-stop (+) DNA after its synthesis. We have observed a similar phenomenon during (−) DNA synthesis in which the tRNA primer molecule was removed from most (−) DNA species greater than 500 to 600 nucleotides but not from all of the (−) DNA species less than this length (6; our unpublished results). Our demonstration of oligoribonucleotides at the 5′ termini of strong-stop (+) DNA is in contrast to previous studies which failed to implicate RNA in the priming of strong-stop (+) DNA (16). However, it is quite possible that these previous efforts to identify such oligoribonucleotides were unsuccessful because most of the oligoribonucleotides had already been removed from the strong-stop (+) DNA synthesized under the reaction conditions employed in these studies or because of the limited resolution of their electrophoretic systems.

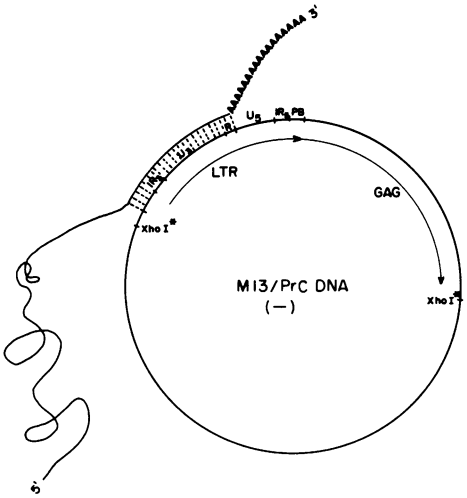

FIG. 5. An 890-nucleotide *Xho*I DNA fragment from Prague C sarcoma virus viral DNA containing a single LTR and molecularly cloned into pBR322 (designated pATV9) was subcloned into a *Sal*I site of M13 mp7, a single-stranded phage. This DNA fragment consists of a complete LTR (IR_3-U_3-R-U_5-IR_5) and the tRNA primer binding site as well as some *gag* gene sequences. A clone designated M13 PrC17 which contained a minus-polarity strand of the viral DNA was identified. Poly(A)-containing 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 (+) DNA. This substrate mimics the natural substrate predicted to be involved in the RNA primer initiation of strong-stop (+) DNA.

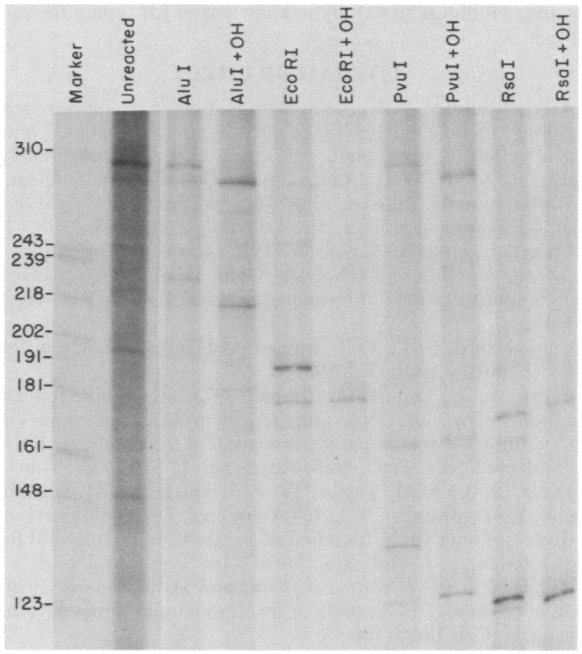

FIG. 6. Restriction mapping of sites downstream from the strong-stop (+) DNA initiation site in the reconstructed reaction and demonstration of alkaline-sensitive species. Plus-sense DNA synthesized as outlined in the text in a reaction with the template depicted in Fig. 5 was digested with restriction enzymes and then subjected to alkali treatment. The DNA product was electrophoresed in 5% polyacrylamide–8 M urea gels. The lengths of 3′-^{32}P-labeled, *Msp*I-digested pBR322 DNA fragments are indicated to the left.

The reconstructed reactions used to synthesize strong-stop (+) DNA gave results very similar to that obtained from detergent-disrupted virus (Fig. 5). The discrepancy between the lengths of the putative oligoribonucleotide primers found with *RsaI* cleavage (approximately 3 nucleotides) and those found with *Pvu*I and *Eco*RI (approximately 10 nucleotides) may be due to an RNase activity in the commercially available *Rsa*I restriction enzyme. The reconstructed reaction contained only an RNA-DNA hybrid template and purified reverse transcriptase, indicating that only these specific components are necessary to facilitate the initiation of strong-stop (+) DNA synthesis. Indeed, preliminary studies from our laboratory have indicated that the reverse transcriptase-associated RNase H activity can cleave the viral RNA genome at the point of strong-stop (+) DNA initiation to provide a 3′ OH which serves as a specific primer for (+) DNA synthesis, thereby providing yet an additional function for the retrovirus RNase H.

The reconstructed reaction described herein provides a useful means for analysis of the initiation of (+) DNA synthesis during reverse transcription as well as several additional intricate steps in viral DNA synthesis. We are currently using this system to analyze the detailed reactions involved and the exact nature of priming of multiple (+)-stranded DNA species.

ACKNOWLEDGMENTS

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