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Initiation of Plus-Strand DNA Synthesis During Reverse Transcription of an Avian Retrovirus Genome

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Two in vitro approaches were used to investigate the priming of strong-stop plus DNA by Rous sarcoma virus. This 340-base DNA species is the first major plus-strand DNA product seen in both infected cells and in endogenous reactions of disrupted virions. In the first approach, we set up a reconstructed system in which strong-stop plus DNA was synthesized by reverse transcriptase from a high-molecular-weight minus-strand viral DNA template. This synthesis was shown to be strictly dependent on the addition of primers to the reaction mixture. The addition of high-molecular-weight RNA from both viral and cellular sources, as well as oligodeoxyguanylate, gave specific synthesis of strong-stop plus DNA, whereas the addition of oligodeoxycytidylate-oligodeoxyadenylate and viral 4S RNA did not. In the second approach, strong-stop plus DNA synthesized in melittin-permeabilized virions was examined on a high-resolution polyacrylamide gel. This DNA was shown to have ca. 11 to 13 ribonucleotides at its 5′ end. These results indicate that strong-stop plus DNA is initiated on a preformed RNA primer.

An early step in the life cycle of retroviruses is the synthesis of a double-stranded DNA intermediate. This intermediate is synthesized by a viral enzyme, reverse transcriptase, with the viral RNA genome as a template. The first strand of DNA synthesized is the minus strand (complementary to the viral genome); this process is initiated with a specific tRNA molecule as a primer (5). Before the minus strand is completed, plus-strand DNA (the same polarity as the viral genome) is initiated, using the minus strand as a template. The first plus-strand DNA seen is a discrete species called strong-stop plus (15). For Rous sarcoma virus, it is about 340 bases in size (19, 23). It is complementary to the 5′ end of minus DNA and contains all the sequences of the long terminal repeat (LTR) seen at each end of the completed linear molecule. A fraction of the strong-stop plus DNA molecules also contains a copy of part of the tRNA which is used to initiate the minus strand (22). In the completed linear molecules of avian retroviruses, the plus strand is fragmented (3, 6), and this likely represents initiation at a discrete set of sites other than that used to make strong-stop plus DNA (7, 9).

The mechanism of initiation of the plus strand has been a long standing question. Initiation of strong-stop plus DNA has been shown to occur at a unique site immediately adjacent to a polypurine tract (14). It has been proposed that primers could be provided by the reverse transcriptase-associated RNase H degradation of the viral genome (23). However, in the in vivo studies of Kung et al., RNA primers longer than 50 nucleotides have not been detected (9). Also, in the in vitro studies by Mitra et al., no RNA has been found attached to the 5′ end of strong-stop plus, as judged by alkali insensitivity of an isotopic 5′ label (14). In contrast, in isotope transfer experiments, Olsen and Watson have detected the presence of RNA-DNA junctions in the plus-strand DNA synthesized in vitro (16), consistent with the use of RNA primers. Although proteins act as primers in some systems (26), we have been unable to detect the presence of a protein primer at the 5′ end of nascent plus strands (7). In the present study we used two in vitro approaches to demonstrate the utilization of RNA primers in the initiation of strong-stop plus DNA.

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MATERIALS AND METHODS

Virus, cells, and reagents. The Prague A strain of Rous sarcoma virus was derived from the transformed quail clone Q-PrA-4 (18). Propagation of this virus in quail embryo fibroblasts was done as previously described (7). Melittin was obtained from Sigma Chemical Co., and placental RNase inhibitor was obtained from Bolton Biologicals. Restriction enzymes were from Bethesda Research Laboratories and were used according to manufacturer's instructions with the addition of 0.5 U of placental RNase inhibitor per μl. Reverse transcriptase purified from avian myeloblastosis virus was kindly provided by the Office of Program Resources and Logistics, Virus Cancer Program, Beltsville, Md.

RNA isolation. To isolate 70S viral RNA, pelleted virions were digested with 0.5% sodium dodecyl sulfate and 0.5 mg of pronase per ml in STE (100 mM NaCl–10 mM Tris [pH 7.5]–1 mM EDTA) for 1 h at 37°C. Nucleic acids were phenol extracted and precipitated with 2 volumes of ethanol. The samples were resuspended and sedimented through a 15 to 30% sucrose gradient in STE for 17 h at 17,000 rpm in a Beckman SW40 rotor. The 70S peak was determined by optical density and was ethanol precipitated. The virus-associated 4S peak was also pooled and precipitated. To isolate viral subunits, the 70S RNA was then denatured at 68°C and rerun on a 5 to 20% sucrose gradient in TE (10 mM Tris [pH 7.4]–10 mM EDTA) for 6 h at 40,000 rpm in a Beckman SW40 rotor. The subunit peak was identified and precipitated as before.

Cellular RNA was isolated by guanidinium isothiocyanate extraction (8) from uninfected quail embryo fibroblasts. It was separated into polyadenylate-plus and polyadenylate-minus fractions by two passages over oligodeoxythymidylate-cellulose.

DNA synthesis. Endogenous reactions were carried out by incubating pelleted virions at a final concentration of 1 mg of viral protein per ml (as determined by UV absorption, in which 1 optical density unit at 260 nm = 158 μg of viral

protein per ml [20]) in the following reaction mixture: 50 mM Tris (pH 8.1), 25 mM NaCl, 25 mM dithiothreitol, 3 mM $MgCl_2$, 1 mM each of the four deoxyribonucleotide triphosphates, 0.5 U of placental RNase inhibitor per μl, and 25 μg of melittin per ml (3). Labeled products were made by decreasing the TTP to 100 μM and adding 4 μM [α-^{32}P]TTP. Reaction mixtures were incubated for 3 h at 41°C, and nucleic acids were isolated by pronase digestion (0.5% sodium dodecyl sulfate and 0.5 mg of pronase per ml for 1 h at 37°C), phenol extraction, and ethanol precipitation.

Large minus-strand DNA was isolated by sedimentation of the endogenous reaction products through an alkaline sucrose gradient (5 to 20% sucrose in 0.33 N NaOH–0.5 M NaCl–20 mM EDTA; Beckman SW40 rotor, 17 h at 32,000 rpm). Gradient fractions were collected, and the samples were glyoxalated (13) and run on 2% agarose gels containing 10 mM phosphate buffer (pH 6.8). After transfer to nitrocellulose (21), strand-specific probes (see below) were used to detect the product. The fractions containing larger minus-strand DNA (2 to 4 kilobases), which was free of LTR plus sequences, were pooled and precipitated with ethanol.

Reconstructed reactions contained 30 mM Tris (pH 8.1), 1% 2-mercaptoethanol, 150 μg of nuclease-free bovine serum albumin per ml, 8 mM $MgCl_2$, and 150 μM each of the four deoxyribonucleotide triphosphates. Templates and primers were added as indicated below. Incubation was for 1 h at 37°C in a final volume of 25 μl. Products were treated with RNase A (100 μg/ml, 1 h at 37°C) and pronase (as above), phenol extracted, and ethanol precipitated. The dried samples were glyoxalated, run on 2% agarose gels, and transferred to nitrocellulose filters.

Bacteriophage M13 DNA containing the minus strand of the LTR was used as a probe as previously described (11).

Enrichment for strong-stop plus DNA sequences. The M13 DNA containing LTR minus sequences was also used to enrich for strong-stop plus DNA from the total endogenous reaction products. The total DNA was cut with *Eco*RI, heated for 5 min at 100°C, and then incubated with the M13 DNA containing the LTR insert (final concentration, 80 to 200 μg/ml) in 10 mM Tris (pH 7.5)–1 mM EDTA–0.5 M NaCl for 2 h at 68°C. The hybridized material was purified by sedimentation of the mixture through a 5 to 20% sucrose gradient in a Beckman SW60 rotor for 90 min at 50,000 rpm. The fastest sedimenting peak of radioactivity, which corresponded in position to the M13 DNA, was pooled and ethanol precipitated.

Sequencing gels. A standard sequencing ladder was generated from the appropriate *Eco*RI fragment of the cloned viral DNA, pSRA2 (4), a gift of W. DeLorbe and co-workers. The Klenow fragment of *Escherichia coli* DNA polymerase was used to label the 3′ ends of the restriction-digested plasmid (10). After a second digestion with *Pvu*II, the 3′-end-labeled fragment spanning the origin of strong-stop plus was isolated by preparative gel electrophoresis. Base-specific cleavage reactions were performed essentially as described by Maxam and Gilbert (12). This sequencing ladder was used as a size standard to examine the endogenous reaction products on a gel (80 cm long) of 6% acrylamide-urea.

RESULTS

Synthesis of strong-stop plus in reconstructed reactions. The basic reaction mixture contained high-molecular-weight minus-strand DNA and reverse transcriptase. Various additions were made to determine which other components might yield specific synthesis of strong-stop plus DNA.

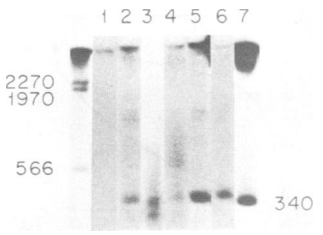

FIG. 1. Reconstruction of strong-stop plus DNA synthesis. High-molecular-weight minus-strand viral DNA containing ca. 0.3 ng of LTR minus sequences was incubated in a reaction mixture with reverse transcriptase and additions as indicated. Products were glyoxalated and subjected to electrophoresis on a 2% agarose gel. After Southern transfer, sequences were detected with a probe for LTR plus-strand DNA. Lane 1, no additions; lane 2, 180 ng of viral RNA subunits added (equivalent to 6.6 ng of LTR sequence); lane 3, 360 ng of viral RNA subunits added; lane 4, 180 ng of viral RNA added, prehybridized to a C_rt of 1 mol · s/liter (that is, 50 μg of RNA per ml in 0.6 M NaCl in TE for 20 min at 68°C); lane 5, 180 ng of viral RNA subunits added, predigested with RNase (7 μg of RNase A per ml in TE for 30 min at 37°C); lane 6, 100 ng of oligodeoxyguanylate added; and lane 7, cloned pSRA2 DNA, digested with *Eco*RI as an LTR size marker. Our virus makes a slightly larger LTR, as seen in the figure. The size markers at the left were *Hin*dIII fragments of phage lambda DNA.

Glyoxalated products were separated by gel electrophoresis and detected with a probe specific for LTR plus-strand sequences. The addition of viral RNA subunits to the reaction led to the synthesis of strong-stop plus DNA (Fig. 1, lanes 1 and 2). The reaction was dependent on the addition of both primers and template (see Table 1), and strong-stop plus DNA was the major discrete product seen. Prior hybridization of the viral RNA to the DNA template was not necessary and in fact seemed to decrease the specificity observed (lane 4). Prior digestion of the viral RNA with RNase A yielded an increased amount of strong-stop plus DNA (lane 5). An increased response was also observed upon the addition of oligodeoxyguanylate (lane 6), which was expected since the sequence immediately adjacent to the point of strong-stop plus DNA initiation is GGGGGA (19). As a higher amount of viral RNA was added, a second product was seen (lane 3). This was ca. 80 bases smaller than strong-stop plus DNA and may correspond to initiation at a site ca. 80 bases downstream, where there is another polypurine stretch (19).

The response curve for added viral RNA is shown in Fig. 2. The amount of strong-stop plus made increased to a plateau level as the concentration of viral RNA was increased. The second species was seen only at higher concentrations. As still higher levels of RNA were added, specificity was decreased, and a smear was observed between strong-stop plus and the smaller band.

A quantitation of the response to various added potential primers is summarized in Table 1, which shows the relative amounts of strong-stop plus DNA synthesized at the plateau level for different added primers. As indicated, several sources of potential primers did not lead to the synthesis of strong-stop plus DNA.

Ribonucleotides at the 5′ end of strong-stop plus DNA. Since the above reconstructions seemed to indicate that RNA was capable of causing the initiation of strong-stop plus DNA, we decided to look for any ribonucleotides still attached to strong-stop plus DNA made in an endogenous reaction. This DNA was labeled during synthesis by the incorporation of [α-^{32}P]-TTP and then digested with

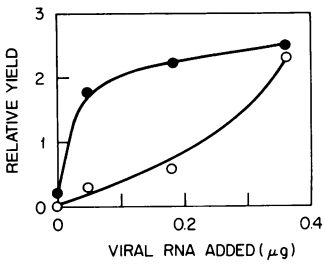

FIG. 2. Dependence of plus-strand DNA synthesis on amount of viral RNA added. Reactions were as described in the legend to Fig. 1, with the addition of increasing amounts of viral RNA. Autoradiograms were quantitated by densitometry to measure the amount of strong-stop plus (closed circles) and the amount of a second smaller species (open circles) made in each reaction.

*Eco*RI. Sequences related to strong-stop plus DNA were selected for by hybridization to LTR minus-strand sequences cloned into M13 DNA. This material was subjected to rate-zonal sedimentation followed by gel electrophoresis of the individual fractions, as shown in Fig. 3A. The value of the enrichment procedure was demonstrated by electrophoresis under denaturing conditions, as shown in Fig. 3B. The DNA which hybridized to M13 DNA (lanes 2 and 3) was qualitatively different from that which failed to hybridize (lane 1). The enriched species, labeled A to D, were further examined by high-resolution acrylamide gel electrophoresis (Fig. 4A). As a size standard to examine these species, we used cloned viral DNA labeled at the 3′ end of the *Eco*RI site within strong-stop plus DNA (see Fig. 4B). This DNA was subjected to base-specific cleavage reactions which are shown in the four lanes on the left. Therefore, the sequence reads from the *Eco*RI site back through the sequences 5′ to the origin of strong-stop plus. The enriched endogenous product, also digested with *Eco*RI, is shown (lane 2). Our identification of bands A to D is summarized in Fig. 4B. Bands C and D were the 3′-terminal *Eco*RI fragments of strong-stop plus DNA which does or does not contain 18 nucleotides of copied tRNA primer (22). Band B was the 5′-terminal *Eco*RI fragment of strong-stop plus DNA, and band A was the same species with additional 5′ ribonucleotides. The evidence for this interpretation was as follows. All four bands specifically hybridized to LTR minus-strand sequences. The lengths of bands B, C, and D were consistent with the published nucleotide sequence (19). Also *Sph*I, which has a single site in strong-stop plus DNA 100 bases from the 5′ terminus, cut bands A and B but not C and D (data not shown). After prior alkali treatment, band A disappeared, and the relative intensity of band B increased (Fig. 4A, lane 1). Our interpretation is that band A represents the covalent attachment of an oligoribonucleotide to the 5′ terminus of nascent strong-stop plus DNA and is a remnant of the priming event. From quantitation of the autoradiogram and normalization (using bands C and D) for total amount of DNA in each lane, we compared the amounts of A and B before and after alkali treatment. This analysis showed that before alkali, ca. 55% of the strong-stop plus molecules had no attached ribonucleotides, and 35% had about 11 to 13 ribonucleotides. With this normalization there was 10% more signal in band B after alkali than in A plus B before alkali. This discrepancy was as much as 40% in another experiment. It could represent a class of strong-stop plus DNA that contains longer, more heterogeneous primers.

TABLE 1. Synthesis of strong-stop plus DNA in response to added primers[a]

Primer added (amt [ng])	Relative synthesis of strong-stop plus DNA
Viral subunits (90)	1.0
Viral subunits (90), prehybridized to DNA[b]	0.8
Viral subunits (90), RNAse digested[b]	3.5
Oligodeoxyguanylate (50)	6.0
Cell RNA, polyadenylate-minus, RNAse-digested (100)	3.5[c]
No additions	<0.1
Viral subunits, reverse transcriptase omitted (90)	<0.1
Viral subunits, DNA omitted (90)	<0.1
Oligodeoxycytidylate (100)	<0.1
Oligodeoxyadenylate (100)	<0.1
Viral 4S RNA (100)	<0.1

[a] Different potential primers were added to reconstructions as in Fig. 1. The amount of strong-stop plus DNA synthesized was determined by densitometry.

[b] Conditions were as described in the legend to Fig. 1.

[c] The response with undigested cell RNA varied from 0.2 to 5.5, depending on the batch, and could reflect the level of degradation of the RNA.

DISCUSSION

We have used two complementary approaches to examine the mechanism used by Rous sarcoma virus for the initiation of strong-stop plus DNA. With the first, we showed that strong-stop plus DNA could be synthesized in a reconstructed system in which viral RNA was added to a high-molecular-weight minus-strand viral DNA and incubated with reverse transcriptase in an appropriate reaction mixture. We

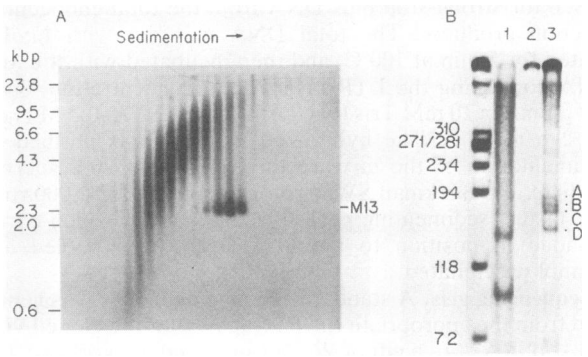

FIG. 3. Enrichment for strong-stop plus sequences. Radiolabeled DNA synthesized by melittin-disrupted virions was digested by *Eco*RI and then hybridized to an excess of M13 DNA containing inserted LTR minus sequences. The hybridized products were sedimented on a sucrose gradient. (A) Gradient fractions were electrophoresed on a 1% neutral agarose gel. The labeled viral DNA that hybridized to the M13 DNA sedimented to the middle of the gradient as shown. The size markers indicated at the left were from *Hin*dIII fragments of phage lambda DNA. (B) Fractions from the gradient were glyoxalated and analyzed by acrylamide gel electrophoresis. Lane 1, material from the gradient that did not hybridize to the M13 with insert; lane 2, material from the gradient that did hybridize to the M13 with insert; and lane 3, a longer exposure of lane 2. The lettered bands at the right are as described in the legend to Fig. 4. The size markers indicated at the left were from phiX replicative form DNA digested with *Hae*III.

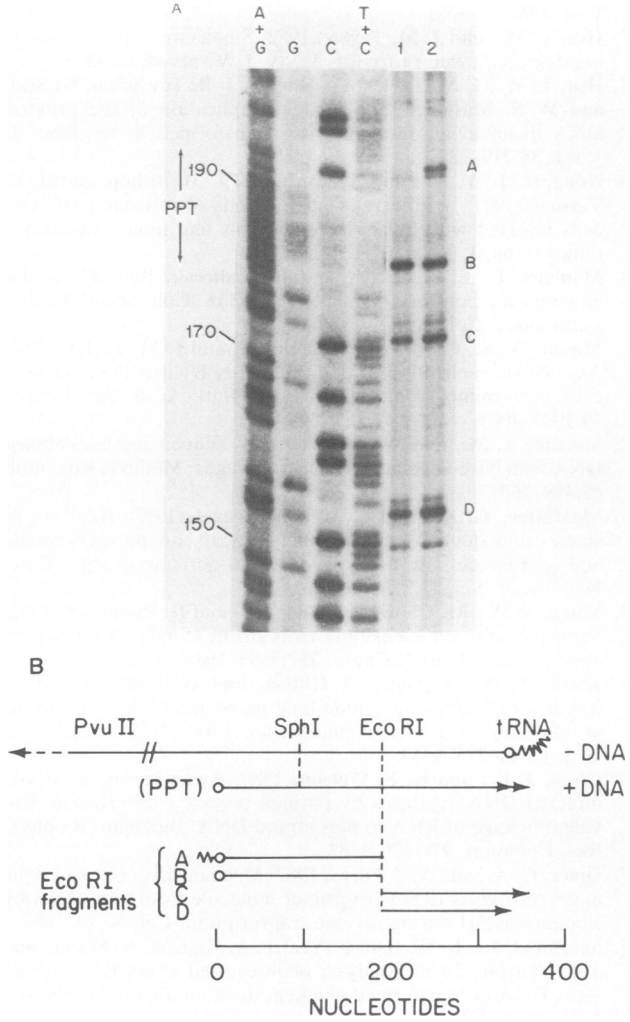

FIG. 4. Ribonucleotides at the 5′ end of strong-stop plus DNA. (A) Labeled strong-stop plus DNA was enriched for as described in the legend to Fig. 3. This was treated with or without alkali (0.3 N NaOH for 10 min at 100°C) and resolved on a gel (80 cm long) of 6% acrylamide-urea. The left four lanes show a sequencing ladder generated from cloned viral DNA labeled at the 3′ end of the *Eco*RI site which includes the origin of strong-stop plus DNA; lane 2, enriched strong-stop plus DNA cut with *Eco*RI; lane 1, DNA as in lane 2 after NaOH treatment. (B) Summary of the positions of the restriction enzymes used on the endogenous reaction products. The expected products of an *Eco*RI digestion of strong-stop plus DNA are indicated as A to D. PPT represents the position that the polypurine tract would occupy in the viral RNA.

used a probe specific for LTR plus-strand sequences because strong-stop plus is known to be the first major plus-stranded species seen in infected cells (23) or endogenous reactions (15). Strong-stop plus DNA was the major discrete species detected even though the probe would have detected any plus-stranded species which included part of the LTR. Prior hybridization of the viral RNA to the DNA template was not necessary. It did not enhance the yield of these reactions, and it even decreased the specificity of initiation. Although it is likely that viral RNA is used in vivo as a source of primers, we found that cellular RNA, after prior RNase digestion, also allowed specific initiation. Our interpretation was that specific initiation may be controlled by the reverse transcriptase or the nature of the template or both and not solely by the primer RNA. Further studies are needed to determine the size and sequence of species within the cellular RNA population that were utilized as primers.

As mentioned earlier, for all retroviruses studied, there is a polypurine stretch in the viral RNA located immediately 5′ to the site at which strong-stop plus DNA initiates (24). That the polypurine tract is involved in specificity of priming was further substantiated by what appeared to be the use of a second purine-rich sequence within the LTR as the concentration of primers was increased. In other work, we have shown that a discrete set of additional plus-strand initiations is used in vivo (21a), and it is of interest to determine whether these are initiated by the same mechanism as strong-stop plus DNA. In the present experiments, the average size of the minus-strand templates was 2 to 4 kilobases. Using still larger minus-strand templates, we hope to be able to examine the initiation of plus-strand DNA at other sites along the genome. Our data are consistent with a polypurine stretch being necessary but not sufficient for specific initiation. Prior digestion of viral RNA with RNase A, which would yield a mixture of oligopurine sequences, did not decrease the specificity of initiation but actually enhanced the yield of strong-stop plus DNA. Also, we tested three purine oligonucleotides. Specific initiation was obtained with oligodeoxyguanylate but not with oligodeoxycytidylate or oligodeoxyadenylate.

In a second approach, we studied the DNA synthesized in endogenous reactions of virions made permeable with melittin. Using high-resolution gel electrophoresis, we demonstrated the presence of a discrete oligoribonucleotide at the 5′ terminus of strong-stop plus DNA. There was some uncertainty in the absolute primer length because of possible differences in migration of ribonucleotides relative to deoxyribonucleotides, especially since the ribonucleotides would all be predicted to be purines and the structure at the 5′ end of the RNA (for example, the state of phosphorylation) was not known. Nevertheless, the deduced length of the RNA oligomer was the equivalent of 11 to 13 deoxyribonucleotides. This size may correlate with the observation that for Rous sarcoma virus (Prague C strain) the sequence adjacent to strong-stop plus DNA is an 11-base purine stretch: 5′-AGGGAGGGGGA-3′ (19). Even though the exact size was not clear, it was evident that there was only one band that disappeared with alkali treatment, so there was no heterogeneity in the primers we detected.

From our data, there were three precise nucleolytic events involved in the priming of strong-stop plus DNA: one to generate the 3′ OH on which DNA synthesis initiated, one to make the precise 5′ end of the primer, and finally one to exactly remove the primer after synthesis was initiated. It was not obvious in which order the first two cuts were made or exactly when they were made relative to the utilization of the RNA as a primer. RNase H, which is an activity of the reverse transcriptase enzyme, hydrolyzes the RNA moiety of DNA-RNA hybrids and is thus a good candidate for the enzyme which makes these cuts. It is known to generate a 3′ OH (1), and the avian enzyme even releases 10- to 12-base oligoribonucleotides (25). This may explain our observed number of ribonucleotides. The specificity we have observed in the first cut appears to be inconsistent with reports that RNase H is a nonspecific exonuclease (25). Perhaps the template itself directs the precise cleavage site. It should be noted that we used inhibitors of RNase A to exclude the possibility that the second cut, which makes the discrete 5′ end on the primer, was due to a contaminating RNase A-like activity, which would release stretches of purines. It remains

to be determined whether the polypurine tract is able to guide the RNase H to make the two initial cuts. It may be relevant that isolated stretches of guanosine ribonucleotides tend to form aggregates in solution (2) so that features other than base pairing may be involved in recognition. In our reconstructions, the synthesis of strong-stop plus was actually enhanced if the viral RNA was first digested with RNase A. This enzyme would release oligopurines with mixed 2′-(3′-)phosphopyrimidines at the 3′ termini. Presumably these would need to be converted to a 3′ OH before utilization as primers. Independent of this requirement for an additional modification, the viral RNA functioned more efficiently in our reconstructions after RNase digestion. This RNase stimulation may simply reflect the fact that large RNAs are more accessible to both the DNA and the relevant enzymatic activities if they have already been reduced to smaller oligopurine tracts.

The third cut mentioned above, that is, the removal of the RNA primer from the 5′ terminus, was not only precise but also efficient. Under our conditions of synthesis, by the time strong-stop plus was completed, as much as 60% of the RNA primer molecules had already been removed. Omer and Faras have shown in a reconstructed system that RNase H readily catalyzes such an RNA removal (17).

In summary, we have shown both in endogenous and in reconstructed reactions that preformed RNA primers were used in the specific initiation of strong-stop plus DNA. The efficient removal of these primers, together with their small size, have probably complicated previous studies aimed at detecting them.

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