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In Vitro Transcription of 70S RNA by the RNA-Directed DNA Polymerase of Rous Sarcoma Virus: Lack of Influence of RNase H

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The influence of Rous sarcoma virus (RSV)-associated RNase H on the in vitro synthesis of DNA by the RSV RNA-directed DNA polymerase was determined under conditions whereby RNase H activity was selectively inhibited with NaF. Not only were we unable to detect any effect on the size, structure, or genetic complexity of the DNA product synthesized in the absence of RNase H activity, but the displacement of DNA from the 70S RNA:DNA hybrid structures was also unaffected. The suitability of 70S RNA:DNA hybrid structures synthesized in vitro to serve as a substrate for RNase H is discussed.

We have been studying the in vitro transcription of the Rous sarcoma virus (RSV) 70S RNA genome in an effort to elucidate the mechanisms by which the RNA-directed DNA polymerase synthesizes DNA. To date, several apparent limitations exist regarding the ability of the DNA polymerase to efficiently transcribe the RSV genome in vitro. These include the relatively small size of the bulk of the DNA product compared with that of the RSV genome, the inefficiency of transcription of RNA into DNA, and the limited transcription of the genome into double-stranded DNA (10, 13, 20). In our attempts to understand the nature of these limitations, and therefore the capabilities of the RNA-directed DNA polymerase, we analyzed the effect of RNase H on the in vitro synthesis of DNA since this particular activity appears to reside on the same polypeptide as the DNA polymerase activity (12, 22, 23) and is thought to play a role in the synthesis of proviral DNA in vivo (2, 7, 11, 13, 15-17). These studies were facilitated by the recent demonstration that the RNase H activity of ether-disrupted virions of avian myeloblastosis virus (AMV) can be selectively inhibited by NaF without appreciably affecting the DNA polymerase activity (2, 7). We obtained similar data employing detergent-disrupted preparations of RSV. For instance, when NaF is included at a final concentration of 30 mM in standard reaction mixtures (2a, 3, 5, 6) greater than 90% of the RNase H activity was inhibited, as determined by solubilization of [^{3}H]poly(rA)$_n$·(dT)$_{12-18}$ (24), whereas the DNA polymerase activity (8) was virtually unaffected. Utilizing this selective inhibition procedure, we compared the nature of the DNA product synthesized in vitro in the presence (−NaF) and absence (+NaF) of RNase H activity. Under reaction conditions that promote extensive transcription of the RSV RNA genome into DNA (2a), no major effects on either the size (a result analogous to that obtained recently by Brewer and Wells with AMV [2]) or the secondary structure of the DNA product can be detected (data not shown).

In addition to studies on the size distribution of the product DNA, we also examined the genetic complexity of the DNA synthesized during NaF inhibition of RNase H activity by two methods. First, the total DNA product was analyzed for its ability to protect radiolabeled 70S RNA from pancreatic RNase hydrolysis to determine the extent of the RSV genome represented by the DNA transcripts (9). The data (Fig. 1) demonstrate that the extent of nucleotide sequence representation of the RSV genome in the total DNA product was unaffected by the selective inhibition of RNase H during enzymatic synthesis of DNA. Secondly, the kinetics of reassociation of denatured duplex DNA was analyzed in an effort to determine the genetic complexity of the double-stranded DNA (21). Similarly, no difference in the reassociation kinetics of the double-stranded DNA products synthesized in the presence or absence of NaF inhibition of RNase H activity was demonstrable (data not shown).

We have also attempted to determine the effect, if any, of RNase H on the integrity of the

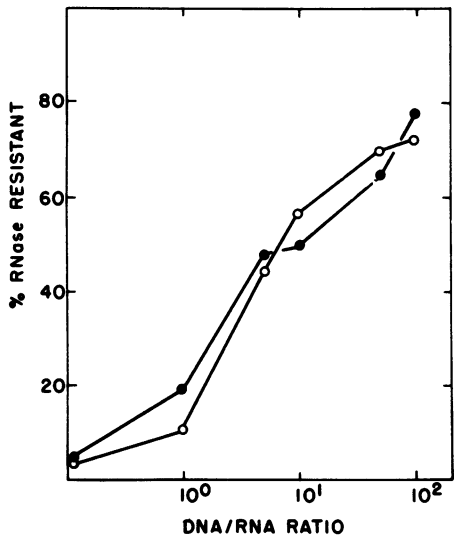

FIG. 1. *Extent of RSV genome represented by RSV-specific DNA synthesized in the presence or absence of RNase H activity. ^{3}H-labeled DNA synthesized by detergent-disrupted RSV in the presence (30 mM) or absence of NaF was purified as described previously (2a, 3). Approximately 0.5 ng (2,200 counts/min) of ^{32}P-labeled RSV 70S RNA (4, 10), 40 μg of yeast RNA, and varying amounts of the denatured ^{3}H-labeled DNA products in 0.1 ml of 3× SSC (1× = 0.15 M NaCl plus 0.015 M sodium citrate) were overlaid with paraffin oil and incubated at 68 C for 36 h. The extent of hybridization was measured by hydrolysis with pancreatic RNase as described elsewhere (3, 9). The results are plotted as a function of the ratio of DNA to RNA present in each sample. Symbols: ●, DNA product synthesized in the absence of NaF; ○, DNA product synthesized in the presence of NaF.*

70S RNA genome and the release of DNA from 70S RNA:DNA hybrid structures during enzymatic synthesis of DNA. These studies were performed with reconstructed reactions containing purified RNA-directed DNA polymerase and 70S RNA (5) to obviate the apparent problem of degradation of template RNA by virion-associated nucleases other than RNase H during enzymatic synthesis of DNA (1, 5, 14, 18, 19). The specific effect of RNase H activity on the 70S RNA template was determined by comparing the profiles, in polyacrylamide gels, of denatured 70S RNA obtained from reactions that lacked deoxynucleoside triphosphates with denatured 70S RNA obtained from enzymatic reactions containing deoxynucleoside triphosphates. Since the 70S RNA template obtained from reactions containing deoxynucleoside triphosphates is in the form of an RNA:DNA hybrid structure, it was conceivable that these structures might provide a suitable substrate for RNase H. However, the profiles of denatured 70S RNA obtained from either of the reactions were quite similar (Fig. 2), indicating that RNase H did not exhibit a dramatic effect on the integrity of the template RNA during the enzymatic synthesis of DNA in vitro.

Finally, we tested the effect of RNase H on the release of the DNA product from 70S RNA:DNA hybrid structures synthesized in vitro since previous pulse-chase experiments performed in our laboratory indicated that DNA initially associated with viral 70S RNA is ultimately displaced from the hybrid structures by some as yet undescribed mechanism (4, 19). We performed similar pulse-chase studies with detergent-disrupted virus under conditions in which RNase H activity is selectively inhibited with NaF. The same amount of pulse-labeled DNA was chased out of the 70S RNA:DNA hybrid structures irrespective of whether or not NaF was present in the reaction mixture (Fig. 3). Therefore, although much of the DNA product may have been displaced from the 70S RNA:DNA hybrids during the course of pulse-chase experiments by the action of other virion-associated nucleases, RNase H did not appear to increase either the rate (data not shown) or amount of DNA released from 70S RNA:DNA hybrids significantly (Fig. 3). This contention was substantiated by the apparent lack of release of DNA from purified 70S RNA:DNA hybrids added to reaction mixtures containing the purified AMV DNA polymerase (Collett and Faras, unpublished data). Furthermore, no displacement of DNA product from 70S RNA:DNA hybrid structures could be detected during the synthesis of DNA in enzymatic reactions containing purified 70S RNA and AMV DNA polymerase (data not shown).

Although the RNase H activity of the RNA-directed DNA polymerase of oncornaviruses is an integral part of the enzyme structure (12, 22, 23) and is probably required to facilitate the synthesis of proviral DNA in vivo, we have not yet been able to detect any significant effect on the synthesis of DNA in vitro. From these studies it appears unlikely that the enzyme activity is responsible for any of the major limitations and restrictions of reverse transcription in vitro. The reasons for our inability to detect any effect of RNase H during DNA synthesis are not clear, but may reflect either the structure of the 70S RNA:DNA hybrid molecules synthesized in vitro or some specific requirement of RNase H activity, or both. For example, since the enzyme is a processive exoribonuclease requiring unblocked termini of the RNA moiety of RNA:DNA hybrids for

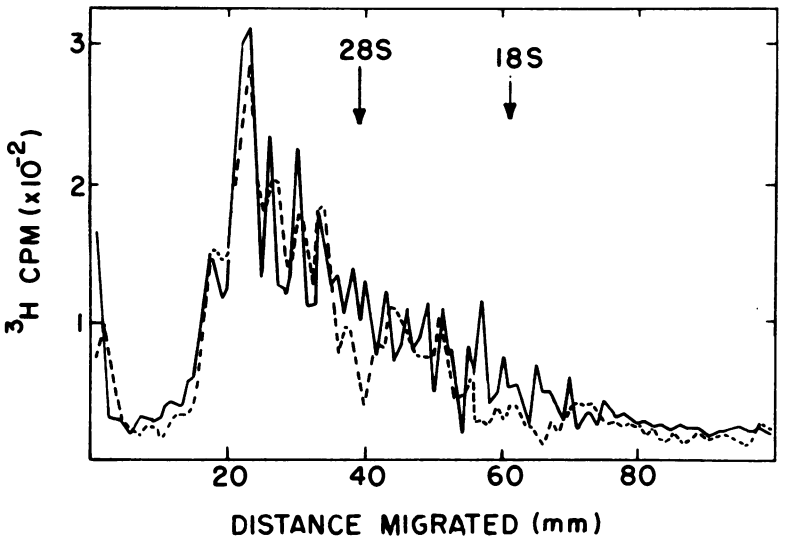

FIG. 2. *Effect of RNase H on 70S template RNA during the enzymatic synthesis of DNA. The effect of RNase H activity on the 70S RNA template during enzymatic synthesis of DNA was determined as follows. Deoxynucleoside triphosphates were omitted from control reactions to inhibit DNA synthesis and thus prevent the formation of a potential RNA:DNA hybrid substrate for RNase H. Conversely, reactions containing deoxynucleoside triphosphates synthesize RNA:DNA hybrid structures, which were tested for their possible utilization by RNase H as a substrate. Both reactions contained ^{3}H-labeled 70S RNA (50,000 counts/min), purified AMV DNA polymerase, and 32 μg of bentonite per ml, which inhibits all traces of nonspecific nucleolytic activity present in the AMV DNA polymerase preparations without appreciably affecting either RNase H or DNA polymerase activity under the conditions employed (Collett and Faras, manuscript in preparation). After incubation at 37 C for 1 h, 70S RNA or 70S RNA:DNA hybrid structures were extracted with sodium dodecyl sulfate-phenol (19), precipitated with ethanol, denatured by heat, and analyzed in 2.25% polyacrylamide gels (0.4 by 10 cm) as described previously (2a, 6, 19). The solid line represents denatured 70S RNA incubated in reaction mixtures lacking deoxynucleoside triphosphates. The broken line represents 70S RNA:DNA hybrids synthesized in reaction mixtures containing deoxynucleoside triphosphates. Differentially labeled 28S and 18S rRNA were included as markers in the gels. The two electropherograms are superimposed for comparative purposes.*

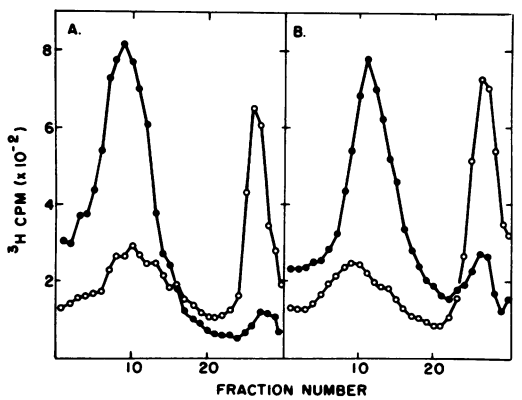

FIG. 3. *Effect of RNase H activity on the displacement of DNA from 70S RNA:DNA hybrids during enzymatic synthesis of DNA by detergent-disrupted RSV. Standard reaction mixtures containing detergent-disrupted virus were incubated at 37 C either in the presence or absence of NaF. Both unlabeled dGTP and dCTP were present at a final concentration of 4 × 10^{-5} M. The labeled precursors [^{3}H]TTP and [^{3}H]dATP were included at a final concentration of 3.5 × 10^{-6} and 1.8 × 10^{-5} M, respectively. Each reaction was divided into two aliquots and incubated for 15 min at 37 C. After the incubation period, one of the reactions (pulse labeled) was stopped by the addition of 0.5% sodium dodecyl sulfate and incubated with 500 μg of Pronase per ml for 30 min at 37 C. The other reaction (pulse-chase) was incubated for an additional 105 min at 37 C after the addition of a 140-fold excess of unlabeled TTP and a 50-fold excess of unlabeled dATP. The reaction was adjusted to 0.5% sodium dodecyl sulfate and 500 μg of Pronase per ml and incubated for 30 min at 37 C. After phenol extraction and ethanol precipitation, samples were analyzed by rate-zonal centrifugation in an SW50.1 rotor for 100 min at 50,000 rpm. A and B represent the enzymatic product synthesized in the absence of NaF and in the presence of the inhibitor, respectively. Symbols: ●, enzymatic product synthesized after a 15-min pulse; ○, enzymatic product synthesized after a pulse-chase.*

activity (12, 16), it is possible that very few, if any, of the 70S RNA:DNA hybrid structures synthesized in vitro exhibit the necessary structure required for activity. However, although

the precise nature of the hybrid structures has not yet been elucidated, we have circumstantial evidence suggesting that the 5′ end of the viral RNA genome might be capable of serving as a substrate for RNase H. First, we and others have localized the principle RSV RNA primer molecule near (within 10%) the 5′ end of the RSV genome (K. Staskus, M. S. Collett, and A. C. Faras, submitted for publication; J. Taylor, in press; and B. Cordell-Stewart, personal communication). Secondly, under the reaction conditions employed in these studies, most of the DNA transcripts ranged between 900 and 1,500 nucleotides in length (2a). Therefore, it is entirely conceivable that the 5′ end of the viral genome is in the form of RNA:DNA hybrid structures suitable as a substrate for RNase H activity. If this were so, then only $\leq$10% of the RSV genome would be affected by RNase II and, therefore, would not be detected in our assay system (Fig. 2). Another difficulty is suggested by the recent demonstration that the 5′ end of the RSV RNA genome is blocked by m^7G and consists of the sequence m^7GpppG^mCp (J. Keith, personal communication). RNase H may be incapable of utilizing this particular terminus as a substrate. Furthermore, avian RNA-directed DNA polymerases may be unable to effectively transcribe the "capped" 5′ terminus of the viral genome in vitro and thus prevent the formation of a suitable substrate for RNase H. Further studies are currently in progress in our laboratory to delineate the nature of the 70S RNA: DNA hybrids with respect to their structure and possible utilization as a potential substrate for RNase H.

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