Specificities Involved in the Initiation of Retroviral Plus-Strand DNA

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Reverse transcription of the retroviral RNA genome begins with tRNA-primed synthesis of a minus-strand DNA, which subsequently acts as the template for the synthesis of plus-strand DNA. This plus-strand DNA is initiated at a unique location and makes use of a purine-rich RNA oligonucleotide derived by RNase H action on the viral RNA. To determine the variables that are relevant to successful specific initiation of plus-strand DNA synthesis, we have used nucleic acid sequences from the genome of Rous sarcoma virus along with three different sources of RNase H: avian myeloblastosis virus DNA polymerase, murine leukemia virus DNA polymerase, and the RNase H of *Escherichia coli*. Our findings include evidence that specificity is controlled not only by the nucleic acid sequences but also by the RNase H. For example, while the avian reverse transcriptase efficiently and specifically initiates on the sequences of the avian retrovirus, the murine reverse transcriptase initiates specifically but at a location 4 bases upstream of the correct site.

Every retrovirus genome that has been studied contains a purine-rich sequence, referred to as the polypurine tract located immediately upstream of the site at which plus-strand DNA synthesis is initiated (19). The same is true even for the plant caulimoviruses, which also replicate by reverse transcription (7), but a somewhat different situation seems to apply to the hepadnaviruses (7). For both avian (8-10, 13, 15, 16) and murine (1, 12) retroviruses, there is evidence that RNA sequences derived from the polypurine tract are used as primers for plus-strand DNA synthesis. For Rous sarcoma virus (RSV), by using the endogenous reaction of detergent-disrupted virions, the predominant RNA primer has been reported to be 12 bases (15) and also 11 bases (10). Reconstructed reactions with purified reverse transcriptase and nucleic acid sequences have been used to obtain similar results (13, 16). How is such specificity achieved? It is apparently not dependent upon any essential sequences downstream of the initiation site (11), whereas upstream of the initiation site, the first 8 nucleotides are not sufficient while the first 28 are (17).

The present study had several aims. To further determine how this primary sequence is relevant to the specificity of plus-strand initiation, the total length of nucleic acid sequences in a DNA-RNA hybrid structure was reduced down to 50 and 40 bases and even to just the 12-base primer region itself. Also, the RNase H-directed cleavages were studied in the absence of reverse transcription. The specificity of the RNase H of the avian reverse transcriptase was contrasted with that obtained from a murine reverse transcriptase and even with the RNase H of *Escherichia coli*. Finally, certain reaction parameters in the combined reactions involving both RNase H cleavage and reverse transcription were examined.

MATERIALS AND METHODS

Enzymes. The purified reverse transcriptases of avian myeloblastosis virus (AMV) and murine leukemia virus (MLV) were obtained from Life Sciences (St. Petersburg,

Fla.) and Bethesda Research Laboratories (BRL; Bethesda, Md.), respectively. The MLV enzyme was synthesized in *E. coli* from the DNA clone pRT601, which expresses the full polymerase sequence along with an additional 12 C-terminal amino acids (4, 5; G. Gerard, personal communication). Quantitatively, it has an RNase H activity comparable to that of the natural polymerase (4, 5), and in studies of the reconstruction of MLV priming, the specificity of initiation was similar to that previously obtained with enzyme isolated from virions (A. J. Rattray and J. J. Champoux, *J. Mol. Biol.*, in press). The RNase H of *E. coli* was also obtained from BRL.

Sources of DNA and RNA. The DNA oligonucleotides corresponding to the minus-strand sequences of RSV were synthesized chemically by using a machine from Applied Biosystems.

Sequences of RSV from -299 to +44, relative to the plus-strand initiation site (+1), were obtained from the clone pATV-GD3A-343 of S. A. Mitsialis (Ph.D. thesis, Columbia University, New York, N.Y., 1983) by using *Bam*HI (BRL) and subcloned into the RNA transcription vector pGem3B (Promega Biotec, Madison, Wis.) to create the construct pG3B-LTR. This clone was linearized at position -134 with *Sph*I (BRL), trimmed with the Klenow fragment of *E. coli* DNA polymerase I (BRL), and then transcribed with the RNA polymerase of phage T7 (BRL) to yield a 186-base plus-strand RSV RNA sequence, preceded at the 5' end by 28 bases of polylinker sequences from the vector. For some experiments this RNA was 5'-end labeled. It was first treated with calf intestinal phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and then incubated with phage T4 polynucleotide kinase (BRL) in the presence of [γ -³²P]ATP (6,000 Ci/mmol; Du Pont Co., Wilmington, Del.).

Determination of RNase H cleavage sites and primer utilization. The hybridization of DNA oligonucleotides to the viral RNA was carried out for 4 h at 68°C in annealing buffer (0.6 M NaCl, 0.04 M Tris hydrochloride [pH 7.4], 0.002 M EDTA). RNase H cleavage reactions were carried out for 1 h at 37°C in 0.1 M KCl-0.01 M MgCl₂-0.05 M Tris hydrochloride (pH 8.0)-0.01 M dithiothreitol-1,000 U of RNasin (Promega) per ml. The final concentrations of RNase H were 500 U/ml (AMV), 4,000 U/ml (MLV), and 25 U/ml (*E. coli*).

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FIG. 1. Nucleic acid sequences used in reconstructions. The 186-base plus-strand RNA was derived from -299 to +44 on the genome of RSV, measured relative to the initiation site of plus-strand synthesis (position +1). The diagram shows only the central region, including the indicated 12-base sequence that acts as the RNA primer (16). Also shown are the three oligonucleotides of minus-strand DNA that were used to test the specificities of the reconstructed reactions. Note that on the 40-base DNA there are 5 bases at each end which will not pair with the template RNA.

To determine RNase H cleavage sites relative to the 5' end of labeled RNA, we used a sequencing gel and an adjacent ladder of partial RNase digests of RNA (2). To determine the sites relative to the 3' end, the cleaved RNA was first treated with RQ DNase I (Promega) to remove DNA oligonucleotides. Then it was subjected to primer extension with a 5'-end-labeled primer (see below) and AMV reverse transcriptase (6). The products were resolved on a sequencing gel relative to a dideoxy-sequencing ladder (14) generated with the same primer on uncleaved RNA.

In some experiments RNase H cleavage was followed by use of the cleaved RNA as a source of primers for DNA-directed DNA synthesis. To do this, dATP, dCTP, and dGTP were added to a final concentration of 500 μ M each along with 1 μ M [α - 32 P]TTP (800 Ci/mmol; Du Pont). After 1 h at 37°C, dTTP was added to a concentration of 500 μ M, and the reaction continued for an additional hour.

RESULTS

An RNase H activity is one that will attack RNA molecules if and only if they are in a hybrid with DNA. The reverse transcriptases of retroviruses contain such an activity (3), and it has been shown that the activity is essential for the replication of the virus (S. Goff, personal communication). Others have used purified reverse transcriptases and shown that the RNase H action will generate fragments 2 to 14 nucleotides long (20). Although no evidence of sequence specificity has been reported, we investigated whether specificity might occur when the RNA substrate contained a polypurine tract, a region where specificity might occur. A 186-base plus-strand RNA was synthesized from the region of RSV that spans the 12-base sequence that acts as the primer of plus-strand DNA synthesis. This RNA was hybridized to one of three different oligonucleotides of minus-strand DNA, each of which included sequences complementary to the full 12-base primer but which had lengths of 12, 40, and 50 bases (Fig. 1). These DNA-RNA hybrids were tested as substrates for three different sources of RNase H activity: AMV, MLV and *E. coli*. Three different methods were used to examine the specificity of the RNase H cleavage.

The first method was use of an RNA substrate labeled at its 5' terminus. The DNA-RNA hybrids were formed and then incubated with the RNase H in the absence of DNA precursors. An electrophoretic analysis of the cleavage product was used to determine the extent of RNA cleavage and locate the cleavage site, in relationship to an RNA sequencing ladder, that was nearest to the 5' end. Typical data are presented in Fig. 2.

When the hybrid of RNA with the 40-mer of DNA was digested with the RNase H activity of the avian enzyme, one main band and one minor band were obtained (Fig. 2, lane

4). From the adjacent RNA sequencing ladder, it was deduced that the major band was produced by a cleavage at the 3' side of the 12-base RNA that normally acts as the plus-strand primer. Similarly, the minor band was produced by cleavage at the 5' side.

When the DNA oligonucleotide that was only 12 bases long was used (Fig. 1), the RNase H activities of both AMV and MLV were essentially unable to cut the RNA in the DNA-RNA hybrid (Fig. 2, lanes 1 and 2). This was not because a hybrid did not exist, since the third RNase H (from *E. coli*) was able to make an efficient cleavage (Fig. 2, lane 3). Incidentally, this cleavage was at the 3' side of the adenosine residue centrally located within the primer sequence. This finding was of additional significance in that it disallowed a quite different but plausible model for how primer specificity was achieved, namely, that the structure of the primer sequence within the RNA of the DNA-RNA hybrid was not even held by Watson and Crick pairing and was therefore "protected," by definition, against any source of RNase H digestion.

As described above, in hybrids with the DNA 40-mer (Fig. 1) the AMV enzyme made cuts, some immediately 5' and most immediately 3' of the primer (Fig. 2, lane 4). This specificity was not achieved with the MLV enzyme, which made some cuts even within the primer sequence (Fig. 2, lane 5). The *E. coli* RNase H demonstrated some specificity, making cuts immediately 5' of the primer sequence, but more than half of the RNAs were actually cut at other locations within the primer sequence (Fig. 2, lane 6).

Different cleavages were obtained with the longest DNA oligomer, the 50-mer (Fig. 1). Presumably because of the increased length, even the AMV enzyme made additional cuts on the RNA 5' of the primer sequence (Fig. 2, lane 7). This was also true with the MLV (Fig. 2, lane 8) and *E. coli* (lane 9) activities. From a comparison of the studies with the AMV enzyme and the 40-mer and 50-mer, it was deduced that the 40-mer was sufficient for efficient cleavage at the 3' site but that the 50-mer was needed for cleavage at the 5' site.

The second method was localization of the cleavage sites relative to the 3' end of the RNA. RNase H cleavage was as described for the first method. Then a DNA primer, labeled at its 5' terminus, was hybridized to the 3' region of the cleaved RNA and extended with avian reverse transcriptase. (Before the primer extension was performed, it was necessary to use DNase I to remove the DNA oligomers used in the RNase H digestion.) A gel analysis of the primer extension products revealed the RNA cleavage site nearest to the 3' end of the RNA and allowed the precise localization of that site relative to an adjacent dideoxy sequencing ladder. Typical data from experiments using both the 40-mer and the 50-mer are presented in Fig. 3.

FIG. 2. Determination of RNase H cleavage sites in relation to the 5' end of the RNA. The 186-base RNA (Fig. 1) was 5'-end labeled, hybridized to one of three DNA oligomers, cleaved by one of three RNase H activities, and then examined on a sequencing gel in comparison with four ladders of partial digestion with RNases (*Bacillus cereus* [Bc], U2, T1, *Phyllobacterium myrsinacearum* [Py]). The DNA oligomers were 12 bases (lanes 1 through 3), 40 bases (lanes 4 through 6), or 50 bases (lanes 7 through 9). The RNase H activities were AMV (lanes 1, 4, and 7), MLV (lanes 2, 5, and 8), and *E. coli* (lanes 3, 6, and 9). The sequence which corresponds to the expected 12-base primer sequence is indicated on the right.

The results obtained with the DNA 40-mer and AMV RNase H showed that most of the primer extensions stopped before the 3' side of the RNA primer sequence (Fig. 3, lane 1). There were several sites, although two seemed relatively more abundant. When AMV was replaced by the RNase H of MLV (Fig. 3, lane 2) or *E. coli* (lane 3), the results again indicated several sites but not the same ones. Similar results were obtained with the 50-mer, except that it detected sites even further 3' relative to the primer sequence (Fig. 3, lanes 4-6).

In a third approach, the RNase H cleavage reactions were set up as before, after which a mixture of radioactive precursors was added in order to assay for specific priming and initiation of plus-strand DNA. After *E. coli* RNase H cleavage, the Klenow fragment of *E. coli* polymerase I was added. Also, since the 12-mer was known to be of a length insufficient to allow RNase H cleavage in most cases (Fig. 2), it was omitted from the set of data. There was also a complication due to the presence of an additional DNA synthesis reaction: the DNA oligonucleotide used in the RNase H cleavage reaction was able to act as a primer in an elongation reaction with the original RNA as template. However the unwanted products were by definition longer

than the DNA oligonucleotides themselves, namely, 40 and 50 bases. The primer extension products were subjected to gel analysis both before and after treatment with alkali to remove covalently attached ribonucleotides. And, as a size control, syntheses in which the RNA was replaced with a second, shorter DNA (5'-AATGTAGTCTTAT-3') that bound as a primer to the larger DNA and created a primer extension product of the precise size expected for correct initiation of plus-strand DNA were also carried out.

In the case of the 40-mer of DNA hybridized to the RNA, and with the AMV enzyme, two major labeled products (less than 40 bases long) were obtained. Their lengths relative to the markers were estimated to be 17 and 30 bases (Fig. 4A, lane 2). When the product was treated with alkali to remove RNA immediately before electrophoresis, the 17-base species remained, the 30-base species disappeared, and a major 18-base species appeared (Fig. 4A, lane 3). Our interpretation was that the correct species was this 18-base species and that after synthesis it must have retained its RNA primer (of approximately 12 [i.e., 30 - 18] bases, as expected). The 17-base species, however, had apparently lost its primer during the reverse transcriptase reaction; thus, if the RNA primer was removed by RNase H, the residual DNA should have a 5' phosphate (16, 20). Such a terminus would be different from both the DNA released by alkali and the 18-base control product, each of which has a 5' hydroxyl group. Further experiments showed that the effect on the electrophoretic mobility of the additional phosphate relative to a hydroxyl group was to make an 18-base species migrate relative to kinased markers as if it were a 17-base species (Fig. 4C).

When the reactions described above were modified from using the AMV enzyme to using the MLV enzyme, there was a major difference in the specificity of initiation (Fig. 4A and B, lanes 4 and 5). As with AMV, there were DNA species with and without an associated primer. However, the MLV enzyme did not initiate at the correct site; initiation was predominantly at 4 bases 5' of the expected correct site. Our interpretation was that the MLV enzyme was unable to correctly recognize the RNA and DNA sequences from the avian retrovirus. Data obtained with the *E. coli* enzymes are shown in Fig. 4A; no labeled species were obtained (lanes 6 and 7). From the earlier studies it was clear that the *E. coli* RNase H could cleave the RNA in a DNA-RNA hybrid (Fig. 2, lane 6 and Fig. 3, lane 3); however, this cleaved RNA apparently was not able to act as a source of RNA primer for the Klenow fragment of *E. coli* DNA polymerase.

The studies described above made use of the 40-mer of DNA. The results were in many ways similar when we used the 50-mer (Fig. 4B). Here the expected size for the correct initiation was 20 bases (Fig. 4B, lane 1). Note that as before, the initiations with AMV enzyme that yielded what seemed to be a 19-base species were actually correct initiations from which the primer RNA had been removed by RNase H (Fig. 4C). (In fact, phosphatase treatment altered the mobility of not only the DNA product from which the RNA primer had already been removed by RNase H but also the DNA product that still had bound RNA primer.) However, a major difference was observed with the *E. coli* enzymes; there was initiation on the 50-mer so as to make a species of about 40 bases. That is, there was initiation and it was at a unique site, but it was not at the appropriate site.

Other potential variables for the specificity of RNase H cleavage were also examined. For the avian system, we tested the effects of monovalent ions (NaCl from 0.075 to 0.150 M) and divalent ions (MgCl from 0.5 to 10 mM),

FIG. 3. Determination of RNase H cleavage sites in relation to the 3' end of the RNA. The 186-base RNA was hybridized to either the 40-base DNA (lanes 1 through 3) or the 50-base DNA (lanes 4 through 6) and then subjected to RNase H digestion with the enzymes from AMV (lanes 1 and 4), MLV (lanes 2 and 5), or *E. coli* (lanes 3 and 6). The cleaved RNA was then extracted (see text), hybridized to a 5'-labeled oligonucleotide (5'-AATGTAGTCTTAT-3'), and then subjected to primer extension with the AMV reverse transcriptase. These products were resolved on a sequencing gel. Uncleaved RNA and the same primer were used as markers, and dideoxy sequencing reactions (14) were carried out (lanes G, T, A, and C). The sequence which corresponds to the expected 12-base primer sequence is indicated on the right.

enzyme concentration (from 100 to 500 U/ml), and reaction temperatures (from 32 to 50°C). Not surprisingly, there were quantitative effects but no qualitative effects on the cleavage; that is, the specificity was not altered (data not shown).

DISCUSSION

In this study we attempted to identify the variables that affect the specificity of initiation of retroviral plus-strand DNA synthesis. We used a 186-base region of the plus-strand RNA sequences from the genome of the avian retrovirus, RSV. This RNA was allowed to interact with minus-strand DNA sequences (12, 40, and 50 bases long) and then used as a substrate for RNase H activity, with or without associated DNA synthesis. Our findings include the following. (i) Even in the absence of DNA synthesis, the avian retroviral RNase H from AMV was able to make specific cuts on the RNA. With the 40- and 50-base DNAs, we found multiple cleavages 3' of the primer RNA and at the 3' and 5' ends of the 12-base primer but not within the primer sequence. Such specificity was not achieved with the murine retrovirus enzyme from MLV or with the RNase H from *E. coli*. (ii) When DNA synthesis was also allowed, the avian reverse transcriptase again yielded significant amounts of the expected specificity of plus-strand initiation. The MLV enzyme initiated 4 bases away from the expected site, consistent with the interpretation that the avian sequences are correctly recognized by the avian enzyme. In other words, both the nucleic acid sequences and the choice of reverse transcriptase are critical for obtaining the appropriate specific initiation of plus-strand DNA synthesis.

From the present studies it is clear that priming specificity is dependent not only upon nucleotide sequence requirements but also upon enzyme requirements. Neither of these alone was sufficient to obtain specificity.

It has been proposed (19; Rattray and Champoux, in press) that the contribution of the RNA sequence to the specificity might not be so much in terms of its exact primary sequence but rather at the level of sequence composition. More specifically, the purine richness might allow interactions between adjacent bases in such a manner as to confer specific resistance to RNase H. Our data show that this interpretation is inadequate; otherwise, the MLV enzyme would have been able to give specific initiation on the AMV RNA sequence.

In terms of sequence requirements, Rattray and Champoux (in press) have recently analyzed the available retrovirus sequences for a consensus for the primer sequence. From this study and from separate base-by-base mutagenesis studies in the range of -6 to +5, they have interpreted the cleavage as being "measured" by the RNase H in relation to a conserved adenosine at position -6 relative to the plus-strand initiation site. Changes at the other positions did not alter the specificity.

As we have reported here, there can be an element of essential compatibility between the source of retroviral sequences and the source of enzyme. We have found that the MLV enzyme has a real but incorrect specificity when it acts on an avian retroviral sequence. (We had missed this in an earlier study which lacked the correct standards [18]).

In conclusion, it is important to stress that the plus-strand priming involves not only the generation by RNase H action of many RNA fragments that could potentially be RNA primers but also the subsequent selection of the correct primer. In the present study, we first used two methods to characterize only the generation of potential primers. We found that many potential primers were generated in addition to those that could be used for correct initiation. Our third series of experiments showed that the selection of the correct plus-strand primer could occur after the generation of RNase H cleavage fragments. In addition, we found that

FIG. 4. Combined generation of RNA primers by RNase H action followed by utilization in DNA-directed DNA synthesis. The 186-base RNA was hybridized to either the 40-base DNA (A) or the 50-base DNA (B) and then subjected to RNase H digestion with the enzymes from AMV (lanes 2 and 3), MLV (lanes 4 and 5), or *E. coli* (lanes 6 and 7). After 30 min we added a mixture of labeled triphosphates with either more reverse transcriptase or the Klenow fragment of *E. coli* DNA polymerase I. After an additional hour, the samples were extracted and analyzed on a sequencing gel either without (lanes 2, 4, and 6) or with (lanes 3, 5, and 7) prior treatment with alkali (16) to remove RNA. Lanes M, 5'-end-labeled DNA markers; lanes 1, reconstruction to create the expected species of plus-strand DNA (see text). (C) Lanes: 1 and 2, as in panel B; 3, portion of the lane 2 material pretreated with bacterial alkaline phosphatase (as previously described [16]); 4, mixture of both treated and untreated samples.

the source of DNA-directed DNA polymerase was no longer relevant; for example, after the generation of primers by AMV reverse transcriptase, it was possible to add the Klenow polymerase along with labeled precursors and obtain specific plus-strand priming (15) (data not shown). Thus, after the generation of primers by RNase H, the selection of the specific primer could be achieved independently of the source of polymerase. In summary, we have shown that

specific primer generation can depend on the appropriate combination of RNA sequence and RNase H, but the subsequent selection of the specific primer can be achieved independently of DNA synthesis or the source of the DNA polymerase. We deduce, as previously proposed (18), that the selection is achieved via base-pairing interactions between the template DNA and the RNase H-generated primer.

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