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When Retroviral Reverse Transcriptases Reach the End of Their RNA Templates

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Luo and Taylor (J. Virol. 64:4321–4328, 1990) have previously shown that when, during RNA-directed DNA synthesis, a retroviral reverse transcriptase comes to a halt at the end of an RNA template, the associated RNase H produces a specific oligonucleotide that contains the 5′ end of that template; in those studies the length of the oligonucleotide was predominantly 17 nucleotides. We have now investigated variables that might affect the formation and length of such a terminal oligonucleotide. We found small but significant variations in the length could be caused by the choice of reaction conditions and also the sources of reverse transcriptase and RNA template. Nevertheless, the general finding in all these situations was that RNase H acted at or about 14 to 18 nucleotides from the 5′ end, thereby supporting the interpretation that in the reverse transcriptase, the cleavage site for the RNase H is held at around this distance behind the DNA polymerase activity. In other words, it appears that for the intact protein, the RNase H and reverse transcriptase activities may work in a coupled or coordinate manner. We also found that more than 80% of the residual 5′ oligonucleotides remained base paired to the RNA-directed DNA product. Furthermore, under certain conditions, these short RNAs could act as efficient primers for an associated DNA-directed DNA synthesis in the reverse direction.

Retroviral reverse transcriptases contain not only a DNA polymerase activity that will copy either RNA or DNA but also a ribonuclease H activity (9, 10, 14, 26). When an RNA template is acted upon by such a reverse transcriptase, there is both the synthesis of a DNA product by the polymerase activity and concomitant endonucleolytic cleavage of the RNA template by the RNase H activity to produce a series of RNA oligonucleotides. We (18) and others (9, 21) have previously described what happens when retroviral reverse transcriptases come to the 5′ ends of their RNA template; the RNase H activity, within the reverse transcriptase, leaves a 5′-terminal RNA fragment that is usually about 14 to 18 nucleotides (nt) in length. In contrast, the oligonucleotides produced when RNase H makes its typical endonucleolytic cuts range from 2 to 26 nt, with the majority being greater than 9 nt (9). Why then is the size distribution narrower for the 5′-terminal fragments?

One reason for the size distribution of the 5′-terminal fragments might be that the oligonucleotide essentially falls off the DNA, thereby rendering itself no longer part of a DNA-RNA hybrid and thus, by definition, no longer a substrate for RNase H cleavage. Another reason might be that the oligonucleotide, even if it remains bound, is too small to be recognized by the RNase H as a substrate. A third explanation, proposed by others (8, 9, 21, 22, 27), is that on the reverse transcriptase, the enzymatic domains for DNA synthesis and RNase H digestion are separated, so that when the polymerase is halted at the end of its RNA template, the cleavage site for RNase H activity is held at a somewhat fixed distance behind that of the DNA polymerase activity. This distance is equivalent to 1.5 to 2 turns of DNA-RNA helix.

In an attempt to distinguish between these three possibilities, we examined the length of the 5′-terminal oligoribonucleotide after reverse transcription, under a variety of experimental conditions, including three different reverse transcriptases and two different templates. Our data support the third model. We also found that even after RNase H cleavage, the majority of the terminal oligonucleotides remained bound to the DNA product. We also found conditions such that these oligonucleotides were able to act efficiently as primers for DNA-directed DNA synthesis to produce double-stranded DNA. This latter reaction could be important for pseudogene formation.

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MATERIALS AND METHODS

Enzymes. The reverse transcriptase of human immunodeficiency virus type 1 (HIV-1), as expressed in bacteria, was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, via Christine Debouck, SmithKline Beecham. Other enzymes were obtained commercially. Avian myeloblastosis virus (AMV) reverse transcriptase was obtained from Life Sciences, and the two murine leukemia virus (MLV) enzymes and the RNase H of *Escherichia coli* were obtained from GIBCO Bethesda Research Laboratories (BRL). The definitions of DNA polymerase units (see Table 4) were as used by the respective suppliers: AMV, 39 U/μg; MLV, 350 U/μg; and HIV, 2 U/μg.

RNA templates and oligonucleotides. RNA templates were transcribed in vitro from expression plasmids, as previously described (18). As summarized in the legend to Fig. 1, the vector was pGEM3Z (Promega) with an insert of sequences derived from a clone of the Prague B strain of Rous sarcoma virus (RSV) (from R. Guntaka). These RSV sequences include part of the long terminal repeat and are numbered relative to the 5′ end of the RNA genome, except that after the 3′ end at 9291, the numbering is continued into U5 (26). Included in the sequence is the polypurine tract (PPT), which is normally involved in plus-strand priming. For RNA synthesis from this construct, RNA polymerases of phages SP6 and T7 were obtained from BRL and used according to the manufacturer's recommendations. To make plus-strand RNA, the construct was first digested with *Hin*dIII and then

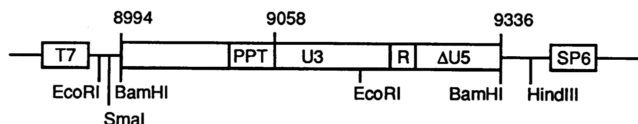

FIG. 1. DNA construct used for RNA synthesis. As explained in Materials and Methods, a cDNA of RSV was inserted into the transcription vector pGem4Z. This insert is shown here in relationship to the promoters for T7 and SP6. To transcribe plus- or minus-strand RNA, the construct was first cleaved with *Hin*dIII or *Eco*RI and then copied with T7 or SP6 polymerase. On the plus strand the features include the polypurine tract (PPT), the unique 3′ region (U3), the small terminal repeat (R), and part of the unique 5′ region (ΔU5).

transcribed with T7 polymerase. In some experiments the construct was cut with *Eco*RI and copied with SP6 polymerase to make minus-strand RNA.

In most experiments we used plus-strand RNA as template and as primer we used an oligonucleotide corresponding to 9186 to 9173. In some experiments minus-strand RNA was used with an oligonucleotide corresponding to 9292 to 9311. For the primer extension studies (see Fig. 3D) we used an oligonucleotide corresponding to 9028 to 9078.

End labeling of RNA templates and DNA primers was carried out with [γ-^{32}P]ATP (6,000 Ci/mM; DuPont) and T4 kinase (GIBCO BRL) by using an exchange reaction in the presence of trace amounts of ADP (19). Labeled species were then purified by electrophoresis and then by electroelution, and about 10,000 cpm was used per reaction.

Reverse transcription and RNase H digestions. Standard reverse transcription buffer contained 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM dithiothreitol, and 100 mM KCl. RNA template (100 ng, 0.8 pmol) and DNA primers (0.5 pmol) were suspended in 20 μl of this buffer, heated at 95°C for 5 min, and then prehybridized at 37°C for 20 min. Four deoxynucleoside triphosphates were then added to 1 mM, along with actinomycin D (Sigma) and reverse transcriptase, as indicated. With preliminary experiments we determined the amount of each reverse transcriptase needed to achieve excess relative to template. The reactions were carried out at 37°C for 1 h; they were followed by extractions with phenol and then with ether and a final collection by precipitation with 2 volumes of ethanol in the presence of 0.2 M sodium acetate (pH 8.1) and 10 μg of dextran, as a carrier, in a final volume of 1.4 ml. It was critical to many of our studies involving RNA oligonucleotides that such ethanol precipitation be used and be efficient. In approximate agreement with Cleaver and Boyer (2), we found that the recovery for species from 5 to 18 nt in length was 25 to 40% relative to that for species longer than 40 nt (7).

The reaction described above is referred to as a one-step reaction. In some cases we used a two-step reaction, as described in the legend to Fig. 2A, in which the first reverse transcriptase was the mutant MLV enzyme that lacks RNase H, after which the extracted product was incubated in a second reaction lacking triphosphates but containing, as indicated, either a nonmutant reverse transcriptase or the RNase H of *E. coli*.

For Fig. 3D, to characterize the synthesis of second strands after a one-step reaction, we treated the product with alkali (0.3 N NaOH, 10 min at 95°C) to remove potential RNA primers. The sample was then neutralized, passed over a small volume of G-50 Sephadex (Pharmacia), and collected by precipitation with ethanol. For primer extension on this DNA we used a 5′-labeled plus-strand oligonucleotide and a Circumvent kit (New England Biolabs).

Digestions with 0.5 U of RNase H of *E. coli* were carried out for 1 h at 37°C in 20 μl of standard reverse transcriptase buffer.

Nucleotide sequencing. RNA sequencing of 5′-end-labeled RNA was as previously described (17). The sequencing ladders were produced by means of partial digestions with alkali and RNases PhyM and T_1. For Fig. 3D, DNA sequencing was carried out with a Sequenase dideoxy sequencing kit (U.S. Biochemicals) by using plasmid DNA and ^{35}S-labeled dATP (DuPont).

Gel electrophoresis. Denatured samples were analyzed on acrylamide gels (of 4, 6, 10, or 20%, as indicated) containing 7 M urea.

RESULTS

Variables that affect the length of the 5′-terminal oligonucleotide. As part of a previous study of reverse transcription (18), we used the reverse transcriptase of MLV to copy to the end of an RNA that was 5′ labeled. As diagrammed in the left side of Fig. 2A, we found that this led to the production of a small, labeled RNA, about 17 nt in length, which apparently remained base paired to the DNA product. We have now carried out a series of experiments to better understand the variables that affect the generation of such an oligonucleotide. Also, as indicated in the right side of Fig. 2A, we have compared the results with those obtained using another strategy, which we call a two-step reaction. The strategy involves a first reaction in which there is RNA-directed DNA synthesis but no RNase H action. This can be achieved by use of a mutant MLV polymerase that lacks the domain for the RNase H. In a second reaction, the action of RNase H from various sources is then tested.

In the initial experiments we carried out such one- and two-step reactions by using as end-labeled template a sequence of retroviral minus-strand RNA, transcribed in vitro with the construct shown in Fig. 1. We tested reaction times of 0.5, 1, 2, and 4 h; this showed that the digestions were incomplete at 0.5 h but virtually complete by 1 h (7). Thus, a reaction time of 1 h was used in all subsequent experiments. The reaction products of RNase H action were separated on a sequencing gel, as presented in Fig. 2B. Also shown are the sequencing ladders provided by partial digestions of the RNA template with alkali and the RNases PhyM and T_1. With these data and the known sequence of the RNA template, we were able to deduce the precise sites of RNase H cleavage. And then, from a densitometric analysis of the autoradiogram, we were able to deduce the relative efficiency of RNase H cleavage at these sites. Such data are presented in Table 1.

In a one-step reaction, the RNase H associated with the MLV reverse transcriptase cleaved at a number of sites, although predominantly at two adjacent sites, nt 17 and 18, respectively. In a two-step reaction, these same preferred sites were used, but the actual abundances were different. The AMV reverse transcriptase under otherwise identical conditions had its own preferred sites of RNase H action, but they were different. For example, most of the cuts were adjacent to nt 14. Likewise, the HIV-1 reverse transcriptase demonstrated yet another set of preferred cleavage sites. As an important control in these studies we used the RNase H of *E. coli*. In a two-step reaction, this enzyme produced fragments of predominantly 11 nt or less. The crystal structure of this enzyme has been determined (13, 28), and it is

even known to be largely similar to the corresponding domain of the HIV-1 reverse transcriptase (4, 6). Even though there are also some notable differences between these structures, we would speculate that one difference potentially relevant to the cleavage specificities as measured here is that the *E. coli* RNase H, unlike an intact reverse transcriptase, does not have the associated protein domains that provide DNA polymerase activity. This difference might be part of the reason why the three reverse transcriptases tested, in both one- and two-step reactions, each gave larger RNase H cleavage fragments. In other words, as in the third model proposed in the introduction, the polymerase domain may provide some control over where the RNase H is able to act.

To understand the basis for these differences in cleavage specificity, we repeated the digestions and analyses for an RNA substrate with a different RNA sequence. This RNA template was derived from the plus strand rather than the minus strand of RSV, as described in the legend to Fig. 1. The present results, as summarized in Table 2, confirm and extend our previously reported results that were limited to studies of only the MLV enzyme. The three reverse transcriptases gave approximately the same cleavages, in that the lengths of the residual oligonucleotides were all about 16 to 18 nt. On closer examination differences could be seen. What is especially relevant is that for each of the three enzymes, the lengths of the oligonucleotides were different when the RNA sequence was changed. That is, all three enzymes demonstrated a sequence preference.

From these studies using three reverse transcriptases, two templates, and two types of reaction, a general picture can be seen in that the associated RNase H activities act at about 14 to 18 nt from the 5′ end. What then is the reason they do not cut closer? It is not because the DNA-RNA hybrid is unstable, because in the control studies with the RNase H of *E. coli*, we found cutting to lengths of 11 nt and less. And, as explained later, this became the rationale for solving another question.

In the above studies we saw no major qualitative differ-

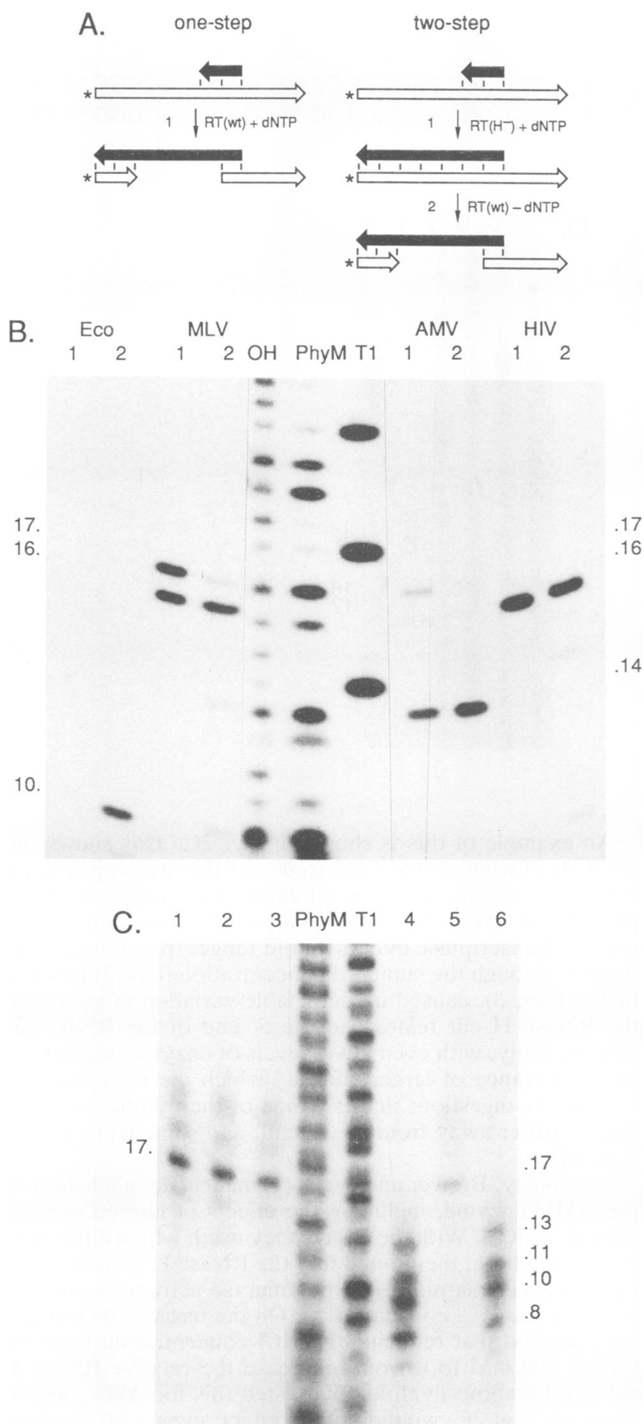

FIG. 2. Two basic reactions with reverse transcriptase. (A) Representations of the two reactions. The RNA template is shown as an open rod, with an indicated 5′ label (*). The oligonucleotide primer before or after elongation by reverse transcription is shown as a solid rod. The left and right diagrams indicate what is referred to in the text as the one- and two-step reactions. In the one-step reaction, we allow the reverse transcriptase to initiate synthesis by using the oligonucleotide primer prehybridized to the labeled RNA template [RT(wt) + dNTP]. This leads to a primer extension product and, because of the RNase H activity with the reverse transcriptase, a digestion of the RNA that has been reverse transcribed. However, as previously shown (18) there remains a short 5′-terminal RNA fragment associated with the DNA product. Also, the 3′ terminus of the RNA remained hybridized to the DNA product, but the extent of this base pairing was not deduced. In the two-step reaction, indicated at the right, the first step is to elongate the primer by using a mutant reverse transcriptase that lacks associated RNase H activity [RT(H$^-$) + dNTP]. In a second step, this product is incubated, in the absence of nucleoside triphosphates, with a reverse transcriptase that does have RNase H activity [RT(wt) − dNTP]. The aim is to examine the 5′-terminal RNA fragment that is produced and compare it with that produced in a one-step reaction. (B) Sequencing gel analysis of residual RNAs, when the template was minus-strand RSV RNA. After one- and two-step reactions, as indicated by lanes 1 and 2, respectively, were carried out as represented in panel A and with details as presented in Materials and Methods, the residual 5′-labeled RNA fragments were resolved on a sequencing gel of 20% polyacrylamide. The enzymes used were *E. coli* RNase H (Eco) or the reverse transcriptases of MLV, AMV, or HIV-1. Also shown is a sequencing ladder provided by the partial digestion of the RNA template with alkali (OH), RNase PhyM (PhyM), or RNase T_1 (T1). (C) Effects of varying the digestion conditions for just one combination: the digestion in a two-step reaction by the RNase H activity of AMV reverse transcriptase. The digestion conditions were as described above (lane 2), with four times more enzyme (lane 3), or with four times less enzyme (lane 1). For lanes 4 to 6, the amount of enzyme was as in lane 1, but KCl was omitted for lanes 4 and 6, and actinomycin D was omitted for lanes 5 and 6. The markers of partially digested RNA are as in panel B.

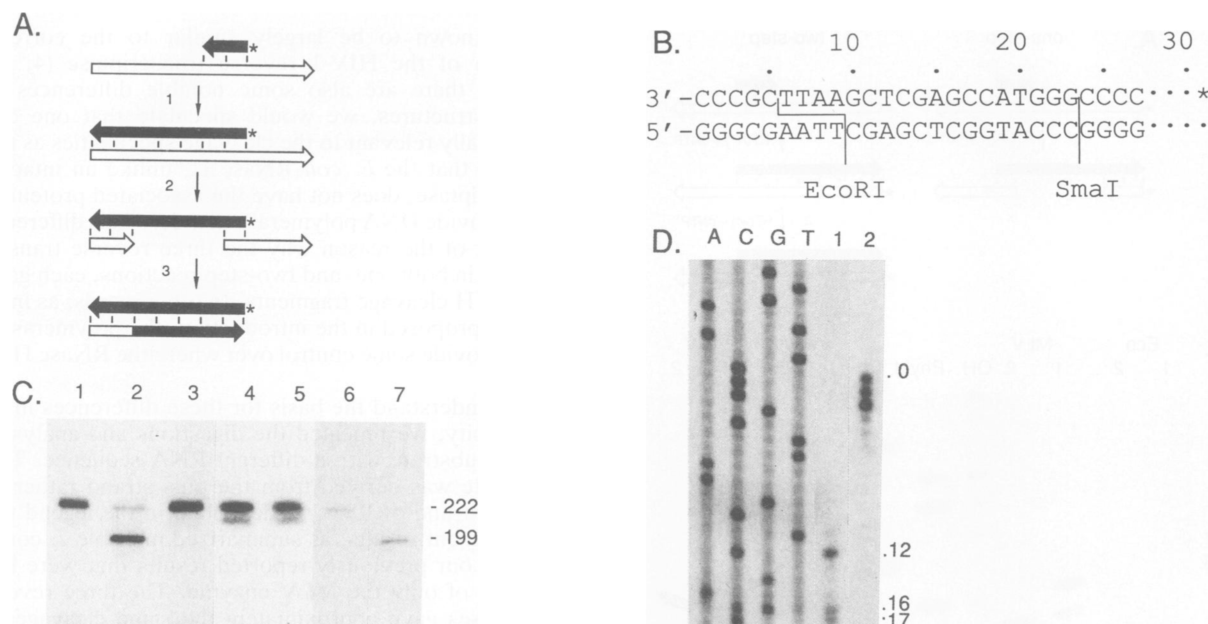

FIG. 3. Utilization of residual RNA as a primer for second-strand DNA synthesis. (A) Indication of how after a primary DNA synthesis on an RNA template, the residual 5′ fragment of that template might go on to become a primer for the synthesis of a second strand of DNA. The conventions are as in Fig. 2A, but it must be noted that now the radioactive label (*) is not on the RNA but on the initial DNA primer. The rationale behind our assay for quantitating second-strand synthesis is as follows. Such second-strand synthesis should make specific parts of the end-labeled primary product susceptible to digestion by certain restriction enzymes. Specifically, as represented in panel B, we expect that if the final product were fully double stranded, then digestion with *Sma*I or *Eco*RI would release fragments of 199 and 217 bases, respectively. (C) Analysis of such digests on a denaturing gel of 4% polyacrylamide along with various controls. All these reactions were carried out in the absence of KCl. The DNA product synthesized with AMV reverse transcriptase on a plus-strand RNA template (Fig. 1) was examined without (lane 1) or with *Sma*I digestion (lane 2) or heat denatured prior to a *Sma*I digestion (lane 3). A product made not with AMV but with a mutant reverse transcriptase lacking RNase H was similarly examined either without (lane 4) or with a *Sma*I digestion (lane 5). A product representing primer extension not on an RNA template but on an otherwise identical DNA template (11) was examined without (lane 6) or with *Sma*I digestion (lane 7). (D) Use of primer extension to determine the origin of second-strand DNA species synthesized in the one-step reaction. Lane 1, primer extension on the second-strand DNA produced in a one-step reaction; lane 2, control of primer extension on a single-stranded DNA template (as in panel C, lane 6). These two products were characterized relative to the dideoxy sequencing ladder (as indicated by C, A, G, and T) obtained with the same primer. In this way, as indicated at the right side, we deduced the positions of the primer extension products relative to the corresponding 5′ end of the original RNA template shown in panel B.

ence in the reaction products for what we call a one- and a two-step reaction. One interpretation might be that the one-step reaction, as carried out by us, is really a two-step reaction; that is, more than one molecule of reverse transcriptase may be involved. For example, one molecule provides the polymerase function, and then at a later time another molecule provides the RNase H function. In order to test the possible relevance of this explanation, we titrated the reverse transcriptase in the one-step reaction.

An example of this is shown in Fig. 2C. This shows one example of what we have referred to as the two-step reaction for AMV enzyme acting on a hybrid containing end-labeled plus-strand RNA. As shown, varying the concentration of reverse transcriptase over a 16-fold range, from 4-fold lower (lane 3) through the standard concentration (lane 2) to 4-fold higher (lane 3), caused no detectable variation in how close the RNase H cut relative to the 5′ end of the RNA. Not unreasonably, with even lower levels of enzyme, we did find the appearance of larger species, which we interpreted as incomplete digestion; that is, some of the hybrids were cut much further away from the 5′ end, and some were not cut at all (7).

Previously, Brewer and Wells (1) made a detailed study of the AMV enzyme, including the effects of altered concentrations of KCl. With the assays they used, which differ from those used here, they found that the RNase H activity could be increased relative to the polymerase activity, simply by reducing the KCl concentration. On the basis of their work, we expected that reducing the KCl concentration from the typical 100 mM to 0 would increase the relative RNase H activity by about fivefold. We tested this for AMV, and as shown in Fig. 2C, we did find an effect; even with fourfold-less enzyme and in the absence of KCl (lane 4), we now observed cutting at several new sites, in the range from 2 to 5 nt closer to the 5′ end of the DNA-RNA hybrid (but certainly not as close as *E. coli* RNase H can cut). This effect was not simply the consequence of more enzyme activity, since as mentioned above, we did not observe such an effect when we increased the relative enzyme concentration by 16-fold (lane 1) relative to that used in the absence of KCl (lane 4). Thus, omission of KCl somehow changed the specificity of oligonucleotide formation by RNase H.

Actinomycin D was typically present in these reactions, with the rationale that it could avoid complications of

TABLE 1. Efficiency of RNase H cleavage sites in relationship to the 5′ end of minus-strand RNA template[a]

Sequence[b]	% Cleaved with following enzyme[c]: MLV One step	MLV Two step	AMV One step	AMV Two step	HIV-1 One step	HIV-1 Two step	E. coli (two step)
20-C	0	0	0	0	0	0	0
19-G	0	0	0	0	0	0	0
18-U	47	17	29	12	5	0	0
17-A	47	46	10	9	84	62	0
16-C	0	0	0	0	5	9	0
15-G	0	0	0	0	2	14	0
14-U	2	16	61	76	0	1	0
13-U	0	2	0	1	0	0	0
12-C	1	0	0	0	0	0	0
11-G	0	0	0	0	0	0	0
10-A	3	14	0	2	4	15	42
9-A	0	0	0	0	0	0	16
8-C	0	5	0	0	0	0	25
7-U	0	0	0	0	0	0	18
6-C	0	0	0	0	0	0	0
5-A	0	0	0	0	0	0	0

[a] An end-labeled minus-strand RNA template (Fig. 1) with added oligonucleotide primer was used in either a one- or a two-step reaction, as introduced in Fig. 2A. The products of RNase H digestion were resolved on an RNA sequencing gel, as in Fig. 2B, and then quantitated by means of a radioanalytic imaging system (AMBIS, San Diego, Calif.). The total radioactivity in species from 5 up to 20 nt in length was considered to be 100%, and the values of the individual species are indicated. Thus, the percentages shown for each species give an indication of the efficiency with which the RNase H cuts in relationship to the 5′ end label on the RNA.

[b] The nucleotide sequence was as expected for the multiple cloning site of the RNA transcription vector pGEM3Z (Promega) which determines the 5′ end of the RNA.

[c] The enzymes used were as for Fig. 2B, in either a one- or a two-step reaction, as indicated. The data indicated at position 17-A, for example, refer to that RNase H cut 3′ of the adenosine at position 17, leading to the release of a 17-base 5′-terminal oligonucleotide.

DNA-directed DNA synthesis (25, 26); however, in the present situation removal of actinomycin D from the reaction mix had no effect (Fig. 2C, lane 5). Removal of both actinomycin D and KCl did have an effect (lane 6). As will be seen in a subsequent section, removal of KCl has other effects on the nature of the reverse transcriptase reaction.

The 5′-terminal oligonucleotide derived from the RNA template remains bound to the DNA product. The results described above suggested to us an easy way to determine whether the 5′-terminal RNA oligonucleotide product of RNase H digestion remained in association with the DNA product. The strategy was simply to isolate the reaction product under nondenaturing conditions and then test the consequences of an additional RNase H digestion, this time with the enzyme of *E. coli*. The rationale was that if it was still bound to the DNA, the RNA would be trimmed to an even smaller size by the *E. coli* enzyme.

An example of this strategy is given in Fig. 4, in which we used the product of a one-step reaction on plus-strand RNA by the reverse transcriptase of AMV. It can be seen that virtually all of the product (lane 1) was still sensitive to a digestion, prior to electrophoresis, with the *E. coli* RNase H (lane 2). The interpretation is thus that the AMV enzyme produced a 5′ oligonucleotide, most of which remained bound to the DNA product. As shown in lane 3, a control was to heat the product prior to the *E. coli* digestion; as expected, this denaturation conferred resistance on the oligonucleotide.

TABLE 2. Efficiency of RNase H cleavage sites in relationship to the 5′ end of plus-strand RNA template[a]

Sequence	% Cleared with following enzyme: MLV One step	MLV Two step	AMV One step	AMV Two step	HIV-1 One step	HIV-1 Two step	E. coli (two step)
20-A	0	0	0	0	0	0	0
19-U	8	1	0	7	11	12	0
18-G	12	9	2	1	3	11	0
17-G	49	77	83	73	22	20	0
16-C	13	9	15	18	54	57	0
15-U	9	2	0	0	0	0	0
14-C	1	0	0	0	0	0	0
13-G	0	0	0	0	0	0	1
12-A	0	0	0	0	0	0	0
11-G	0	0	0	0	0	0	10
10-C	0	0	0	0	0	0	16
9-U	0	0	0	0	0	0	17
8-U	0	0	0	0	0	0	18
7-A	0	0	0	0	0	0	19
6-A	0	0	0	0	0	0	0
5-G	0	0	0	0	0	0	0

[a] The method of evaluation was as described in the footnotes to Table 1. The only experimental difference is that here we used a plus-strand RNA and its primer rather than a minus strand.

A quantitation of the data shown in Fig. 4 revealed that 95% of the terminal oligonucleotides produced by AMV were still susceptible to a further digestion with the *E. coli* RNase H. Thus, we infer that 95% of these terminal oligonucleotides had remained bound to the DNA product strand. We then carried out a series of similar experiments to determine what would happen with different templates and different sources of reverse transcriptase. As summarized in Table 3, we examined three sources of reverse transcriptase acting on two different RNA templates. In all cases the fraction of the 5′-terminal oligonucleotide that remained in association with the DNA product was at least 80%. With this result, we were prompted, as described in the next section, to go on and ask whether such an oligonucleotide

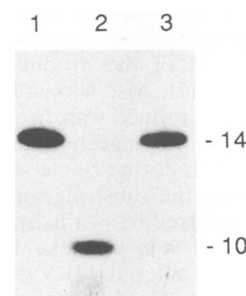

FIG. 4. Evidence for association of residual RNA with the DNA product. After a one- or two-step reaction, such as described in the legend to Fig. 2A, the residual labeled RNA fragment will be sensitive to a further digestion with the RNase H of *E. coli* if and only if it remains still bound as a DNA-RNA hybrid to the DNA product. Lanes 1 and 2, such fragments before and after a second digestion; lane 3, negative control in which the fragments were denatured prior to the RNase H digestion. The products were resolved on a denaturing gel, but rather than use the sequencing gel, as in Fig. 2B, we used a smaller and more convenient 10% polyacrylamide gel.

TABLE 3. Susceptibility of oligonucleotides created with reverse transcriptase to a second digestion with *E. coli* RNase H[a]

RNA template[b]	% Susceptible following synthesis with[c]:		
	MLV	AMV	HIV
Minus strand	97	80	96
Plus strand	>99	95	90

[a] Reactions were carried out as for Fig. 3.
[b] These two RNA templates are as described in the legend to Fig. 1.
[c] A total of six reactions were carried out, with three reverse transcriptases, as indicated, and two templates. For each reaction, aliquots of the product were tested for susceptibility to a further digestion with the RNase H of *E. coli*, and then quantitated by using a radioanalytic imaging system, to determine the percentages, as indicated.

TABLE 4. Quantitation of the ability of primary primer extension products to proceed on to second-strand DNA synthesis

Reaction conditions[a]	% Cleaved[b]
KCl (mM)	
0 (standard reaction)	53
25	47
50	24
100	<1
$MgCl_2$ (mM)	
10 (standard reaction)	56
5	46
2.5	4
RTase (U)	
5 (standard reaction)	65
10	81
20	87
RTase	
AMV (standard reaction)	65
MLV (50 U)	97
HIV-1 (0.25 U)	>97
Actinomycin D	
None (standard reaction)	65
100 μg/ml	48

[a] The standard 20-μl reaction mixture contained plus-strand RSV RNA, end-labeled oligonucleotide primer, and AMV reverse transcriptase (RTase). Both KCl and actinomycin D were omitted. The reaction was for 1 h at 37°C.
[b] The fraction of the primary first-strand product that went on to act as a template for second-strand DNA synthesis was determined by means of quantitating, with a radioanalytic imaging system, the susceptibility of the primary product to a *Sma*I digestion, as in Fig. 3B.

could then in a subsequent reaction act as an RNA primer for a second strand of DNA.

Utilization of the 5′-terminal oligonucleotide to synthesize a second DNA strand. We reasoned that if the terminal oligonucleotide produced during RNA-directed DNA synthesis were to be able to then act as a primer for DNA-directed DNA synthesis, it would be necessary to modify the reaction mixture. First of all, we chose to remove the actinomycin D, since it has been reported to be an inhibitor of DNA-directed DNA synthesis by reverse transcriptases (9, 26). Secondly we chose to 5′ label not the RNA template but the oligodeoxyribonucleotide primer. And as represented in Fig. 3A, we reasoned that if second-strand synthesis occurred (step 3), it would lead to a product of which parts would consist of double-stranded DNA. These parts would thus make the primary end-labeled product susceptible to digestion with certain restriction enzymes. In fact, the fraction of full-length first strands that could be so cleaved should be a precise measure of the molecules that went on to act as templates for second-strand synthesis. Two specific restriction enzymes were chosen from the known sequence of the plus-strand RNA template (Fig. 1). As shown in Fig. 3B, there is a potential *Sma*I site 23 nt from the 3′ terminus of the initial 5′-labeled primer extension product. Secondly, there is on this sequence a potential *Eco*RI site only 5 nt from the 3′ terminus. After such digestions the products were denatured and examined by gel electrophoresis.

An example of this strategy is shown in Fig. 3C. About 80% of the 222-base first-strand product (lane 1) was cleaved by *Sma*I to release a 199-base species (lane 2). In contrast, no detectable amount of the product was susceptible to digestion with *Eco*RI (7). Also shown in Fig. 3C are several controls. When the product was heat denatured prior to digestion with *Sma*I, no digestion product was obtained (lane 3); this proved, first, that single-stranded DNA was not cut and, second, that the substrate for the cut product was not of a structure referred to as a hairpin or snap-back form (25). Another control was to make the primer extension product by using the altered MLV reverse transcriptase, which lacked RNase H. This product was 222 nt in length, as expected (lane 4), and was insensitive to digestion with *Sma*I (lane 5); this proved that DNA in a DNA-RNA hybrid structure could not be digested with *Sma*I. Yet another control required making a product that was double-stranded DNA along its entire length. This was done by carrying out the primary synthesis on a template that was not RNA but DNA. Such a product (lane 6) was 222 nt in length, as expected, and was sensitive to digestions with *Sma*I (lane 7).

With this assay it was possible to readily deduce the efficiency with which second-strand DNA synthesis was initiated. However, this approach did not yield a correspondingly good indication of where second-strand synthesis was initiated from. For example, the susceptibility to *Sma*I coupled with a resistance to *Eco*RI indicates that synthesis of the second strand initiated somewhere between 11 and 20 bases from the 3′ terminus of the primary DNA. This calculation allows for the fact that the two restriction enzymes have a 6-base recognition sequence. Now since all known reverse transcription initiation events depend upon some form of primer, usually an RNA (9, 26), we would interpret our data as circumstantial evidence that the primer for the second strand was actually an RNase H cleavage fragment derived from the 5′ terminus of the original RNA template.

In order to obtain a better indication of where the second-strand synthesis was initiated, we made use of an alternative approach. As described in Materials and Methods, we treated the product with alkali to remove putative RNA primers and then carried out a primer extension reaction. As shown in Fig. 3D, lane 1, we observed not one but several primer extension products. These products were all about 10 to 18 nt shorter than extension to the 5′ end of a full-length control template (lane 2). And, using a dideoxy sequencing ladder, we were able to infer the sites at which second-strand DNA synthesis was initiated. These locations, as indicated at the right side of Fig. 3D, were in fact very similar to the RNase H cleavage sites that we had previously mapped on the RNA template (Fig. 2C, lane 6). (The positions differed by no more than 2 nt, and even this discrepancy could probably be explained by an apparent limitation of the primer extension reactions, as indicated in Fig. 3, lane 2, where some of the products stopped one or even two

nucleotides prior to the true 5′ end of the template.) Thus, we interpret the similarity of the two results as evidence that the 5′-terminal oligoribonucleotides produced by RNase H action are able to go on and act as primers for second-strand DNA synthesis.

We also applied the original assay system, as presented in Fig. 3C, to examine the conditions that affect the efficiency of such second-strand DNA synthesis. A summary of such experiments is presented in Table 4. As shown, this reaction was more efficient in the absence of KCl; in fact the standard reaction conditions, as shown in Fig. 3B, lanes 1 to 3, involved the omission of KCl. As seen in Table 4, the addition of increasing amounts of KCl led to an inhibition of second-strand synthesis. In contrast, raising the concentration of AMV reverse transcriptase increased the efficiency. Suboptimal concentrations of $MgCl_2$ had an inhibitory effect. Other reverse transcriptases could replace AMV; for example, with the enzymes of HIV-1 and MLV, the efficiency was at least 97%. Finally, actinomycin D, which is generally considered to be an inhibitor of DNA-directed DNA synthesis by reverse transcriptase (9, 26), did show a reproducible effect. However, the effect was only small; the bulk of the initiation of second-strand synthesis was in fact resistant to the presence of actinomycin D. This latter result is the subject of further studies on the action of reverse transcriptase inhibitors (7).

DISCUSSION

Others have presented evidence that the polymerase and RNase H activities of reverse transcriptase can act as if coordinated. The most dramatic in vivo example is during hepadnavirus minus-strand DNA synthesis, when it is possible to observe that minus strands extracted from inside infected cells have been synthesized by the simultaneous elongation of the nascent DNA and the degradation of that RNA which has just been reverse transcribed (23). Others have studied this in vitro by using specially defined templates and primers (3, 15, 16). We have addressed this phenomenon, in vitro, at a specific point in reverse transcription, namely, at the point where the polymerase reaches the end of the RNA template. We have shown that a residual oligonucleotide of RNA is not only created by RNase H action, but it is left in place. Our results confirm and extend previous studies by Oyama et al. (21). What we have found is that this oligonucleotide can be heterogeneous in length, with an average size ranging from 14 to 18 nt. We examined a number of parameters to see how they might affect the lengths of these oligonucleotides. We found no effect when the enzyme concentration was altered over a 16-fold range. Nor did we find an effect if the reaction were carried out in what we described as a one- or two-step reaction, that is, cleavage with or without associated DNA synthesis. However, a number of parameters did affect the length of the oligonucleotides. Reducing the KCl in the reaction mixture allowed the enzyme to cut several nucleotides closer to the 5′ end of the RNA; presumably this occurred via a relaxation of the interaction between the reverse transcriptase and the substrate. Additionally, when we compared reverse transcriptases from three different species of retroviruses or used different RNA templates, we found small but real differences in the cleavage site. Nevertheless, in all these studies, the retroviral RNase H activity made cleavages at around 14 to 18 nt from the 5′ end of the RNA. We would conclude that our data are best explained by the third model proposed in the introduction. That is, for the three reverse transcriptases studied, the cleavage site determined by the RNase H domain is held at this somewhat fixed distance behind the DNA polymerization site. According to this model, the polymerization site was dominant relative to the RNase H domain because, even in our two-step reaction, when RNase H action occurred after DNA synthesis, the same fixed distance was maintained. Furthermore, the removal of KCl, which led to a shortening of the oligonucleotide length, could be interpreted as a relaxation of this specificity.

Our results indicate not only that these 5′-terminal oligonucleotides are produced but also that >80% remain bound to the 3′ end of the DNA. This is in contrast to the models of retroviral reverse transcription, where it is generally represented that prior to template switching, RNase H action renders the minus-strand strong-stop DNA completely free of hybridized RNA template (26). Since we have previously shown that in the presence of an acceptor template, such switching can still occur with high efficiency (18), we would suggest a revised model, that is, that the switch to the other template involves a form of progressive exchange of base pairing, a strand exchange involving the DNA product, the terminal oligonucleotide, and the acceptor template. Such an exchange might be energetically more favorable than what is assumed in the current models, namely, the one-step breaking and then the reformation of base pairing involving 15 to 18 nt.

We have also shown that under appropriate conditions, such residual RNA oligonucleotides can go on to act as primers for DNA-directed DNA synthesis. We evaluated several parameters for such second-strand synthesis and found conditions that yielded efficiencies above 80%. Such conditions could be of real practical value to those who wish to make double-stranded cDNAs. However, in terms of speculations of biological relevance, a primary concern is that the reaction may be somewhat artifactual; it did not occur efficiently when the concentration of KCl was increased (Table 4) to even less than 139 mM, which is considered to be the concentration present in the normal cellular environment (5). In fact, our data may be interpreted as evidence that such priming events may be suppressed in vivo. Nevertheless, consider the following two examples. First of all, during reverse transcription of the hepadnavirus RNA into double-stranded DNA, there is a priming event for second-strand DNA synthesis which is in some ways like what we have described here; a 19-nt fragment from the 5′ end of the RNA template, presumably by RNase H action, becomes the primer for such synthesis (15, 20). The complication is that in most cases this primer, by a mechanism that is not yet understood, is somehow translocated so as to act for DNA synthesis at another site. It is only in a small fraction of priming events (15, 20) or as a consequence of alterations on the viral RNA (3, 16) that the primer acts at the site at which it was produced to carry out what has been referred to as in situ priming. A second example might be in the case of retroviruses. However, although there is documented priming of plus-strand DNA by RNase H-derived RNA fragments, such priming apparently does not occur at the 5′ end of the template but at one or more specific internal sites associated with purine-rich sequences in the RNA primer (e.g., PPT in Fig. 1) (12, 24, 27).

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