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Template Switching by Reverse Transcriptase during DNA Synthesis

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The ability of reverse transcriptase to make template switches during DNA synthesis is implicit in models of retrovirus genome replication, as well as in recombination and oncogene transduction. In order to understand such switching, we used in vitro reactions with purified nucleic acids and enzymes. The assay system involved the use of an end-labeled DNA primer so as to allow the quantitation of elongation on a donor template relative to the amount of elongation achieved by template switching (by means of sequence homology) when an acceptor template RNA was added. We examined several variables that affected the efficiency of the reaction: (i) the reaction time, (ii) the relative amounts of acceptor and donor template, (iii) the extent of sequence overlap between the donor and acceptor templates, and (iv) the presence or absence of RNase H activity associated with the reverse transcriptase. The basic reaction, with RNA templates and normal reverse transcriptase, yielded as much as 83% template switching. In the absence of RNase H, switching still occurred but the efficiency was lowered. Also, when the donor template was changed from RNA to DNA, there was still switching; not surprisingly, this was largely unaffected by the presence or absence of RNase H. Finally, we examined the action of the RNase H on RNA templates after primary transcription but prior to template switching. We found that in most cases, both ends of the original RNA template were able to maintain an association with the DNA product. This result was consistent with the work of others who have shown that RNase H acts as an endonuclease.

In the life cycle of a retrovirus, the RNA genome is reverse transcribed initially into a minus-strand DNA, which in turn acts as a template for the synthesis of a plus-strand DNA (18). The syntheses of each of these DNA strands are similar in that they are initiated from a unique location on a specific RNA primer molecule. Also, both syntheses soon reach the end of their templates and come to what is called a strong stop. According to the model, there then occurs a base-pairing interaction between the 3′ end of the strong-stop DNA and a complementary RNA sequence. Such an interaction effectively allows template switching and, thus, extension of the strong-stop species by DNA synthesis at a second location. The RNase H activity that is normally part of the reverse transcriptase molecule does play a part in this template switching; it is thought to degrade the relevant RNA in the original hybrid with the strong-stop species, thereby freeing that DNA so that it may make the necessary base-pairing reaction with the second template site.

There are additional situations in which a related form of template switching is considered relevant to retrovirus replication.

The second is in the copy choice model of recombination proposed by Coffin (1; J. M. Coffin, Appl. Virol. Res., in press). The copy choice model is an extrapolation from the above-mentioned template switches that occur for minus- and plus-strand DNA synthesis. It proposes that single-stranded nicks in the retrovirus genome would create new strong-stop species of minus-strand DNA during reverse transcription; these species could then participate in template switching to the homologous site on a second copy of viral RNA, especially since the viral particles are thought to contain a pair of viral RNAs held in a so-called dimer linkage structure. By such template switching during reverse transcription, a single full-length minus-strand DNA per virion could be produced even if both of the RNAs contained many single-strand nicks. Also, if the two RNAs were different, then efficient recombination would be observed. Hu and Temin (6) have recently established this requirement for such a heterodimeric RNA, so as to allow high-frequency retrovirus recombination. Also, studies by Goodrich and Duesberg (4) support the model of Coffin. The data do not address a quite different model of recombination, referred to as displacement-assimilation, proposed by Junghans et al. (7).

There is a third proposal invoking template switching during reverse transcription. Swanstrom et al. (17) have proposed a model to explain the ability of retroviruses to transduce cellular proto-oncogene sequences. This model invokes template switching between cellular and viral RNA sequences during reverse transcription.

In order to get a better understanding of template switching and how it works, we set up a model in vitro system with purified templates and enzymes, in which the switching could be quantitated and characterized. Our findings have significantly improved our understanding of the phenomenon along with the mechanism of action of the RNase H.

MATERIALS AND METHODS

Enzymes. Two different forms of cloned reverse transcriptase were purchased from Bethesda Research Laboratories. The first was the intact enzyme of Moloney murine leukemia virus (MLV) (3), while the second contained a C-terminal deletion, so as to remove the RNase H activity (8). The RNase H of *Escherichia coli* was also purchased from Bethesda Research Laboratories.

RNA and DNA sequences. The sequences chosen as templates for reverse transcription were from the genome of Rous sarcoma virus. (In retrospect, there was no significant reason for using these sequences over any others.) The sequences were derived from a Rous sarcoma virus (Prague

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B strain) provirus subclone. It spanned the region from nucleotide −299 to +44, by the convention that +1 corresponds to the first nucleotide of the R sequence; that is, +1 is equivalent to the 5′ nucleotide of the RNA genome. To make a donor RNA template, this region was subcloned into the RNA transcription vector pGem3B (Promega Biotec). This clone was cut with *Hin*dIII prior to transcription with phage T7 RNA polymerase. The resultant RNA contained 28 bases (b) of 5′ vector sequences. It was gel purified and then cleaved as follows. A 13-b oligonucleotide complementary to −245 to −233 was hybridized, the RNA was digested with the RNase H of *E. coli*, according to the instructions of the supplier, and then the resultant 315-b 5′ fragment was extracted and gel purified. To make the standard RNA acceptor template, a second pGem3B subclone was made, spanning −299 to −140. This vector was cut with *Sph*I prior to transcription with T7 polymerase, and then the RNA product was gel purified. For the overlap experiment shown in Fig. 2C, we had to further modify the donor RNA to reduce the overlap with respect to the acceptor template. This was done, as above, with specific oligonucleotides and RNase H digestion.

To generate plus-strand DNA templates, the above-mentioned plasmid clones were digested with either *Hin*dIII or *Sph*I. Then, together with a single oligonucleotide, the DNA was used as template for asymmetric polymerase chain reactions (5) to generate single-stranded DNA. The resultant donor template spanned −245 to +44 (plus 30 b of 3′ vector sequence). The acceptor DNA spanned −299 to −140 (plus 17 b of 5′ vector sequence). Again, the products were gel purified prior to use.

Reverse transcription. Synthesis reactions were carried out in the presence of 4 deoxynucleotide triphosphates (1 mM each), 10 mM $MgCl_2$, 100 mM KCl, 10 mM dithiothreitol, 50 mM Tris hydrochloride (pH 7.8), 1,000 U of RNasin (Promega Biotec) per ml, and 50 U of reverse transcriptase in a 10-μl reaction volume. The reaction mixtures also contained 200,000 cpm of DNA primer that had been 5′ end labeled with phage T4 kinase (Bethesda Research Laboratories) and [γ-^{32}P]ATP (6,000 Ci/mM; Du Pont, NEN Research Products). This primer was 14 b long, spanning the region −119 to −106, and complementary to the donor templates. The RNA templates were added at approximately 30 ng per reaction (unless otherwise indicated), so that the molar ratio of template to primer was approximately 15. The amounts of the DNA template used per reaction were significantly lower. For reactions using only RNA templates, we also added 50 μg of dactinomycin (Sigma Chemical Co.), to inhibit DNA-directed DNA synthesis. The reaction volumes were 10 μl, with incubation at 37°C for 1 to 3 h, unless otherwise indicated. In some cases, the DNA products were treated with alkali (0.3 N NaOH, 10 min, 95°C) to remove RNA templates prior to gel analysis.

For the experiment shown in Fig. 6B, samples were treated prior to electrophoresis with calf intestinal phosphatase (Boehringer Mannheim Biochemicals). The treatment was 0.1 U in 10 μl for 1 h at 37°C, with the buffer recommended by the supplier.

Gel analysis. Denaturing and nondenaturing gels of 6% polyacrylamide were run as described by Sambrook et al. (14). After electrophoresis, gels were dried onto DEAE paper (DE-81; Whatman, Inc.) and then subjected to autoradiography in the presence of one intensifying screen. Alternatively, in order to get precise quantitation, the dried gel was analyzed in two dimensions with an AMBIS radioanalytic imaging system.

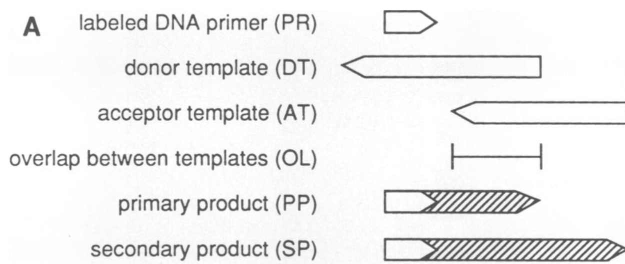
RESULTS

In order to study template switching, we set up the in vitro assay system, as summarized in Fig. 1A. DNA synthesis was initiated on a 14-b DNA primer (PR) 5′ end labeled with ^{32}P. The primer could base pair with the donor template (DT) but not to the acceptor template (AT). The two templates shared a region of sequence identity, referred to as the overlap. During reverse transcription, the primer was first extended to the end of the donor template molecule to create what is called the primary DNA product (PP). And in the presence of the acceptor template, the reverse transcription of some of these primary products switched to the acceptor template, extended to the end of that template, and thereby created what is called the secondary product (SP). Fig. 1B gives an example of how the PP was resolved from SP and PR by using electrophoresis into an acrylamide gel. This gel was dried and subjected to autoradiography, as shown, or subjected to direct quantitation in two dimensions by using an AMBIS radioanalytic imaging system. For Fig. 1, lane 3, we thus deduced that 83% of the DNA products made use of template switching. The reverse transcriptase used in this example was synthesized in bacteria from a molecular clone of the reverse transcriptase of MLV (3).

Figure 2 summarizes some of our results concerning three variables that affect the efficiency of template switching. In these studies, the donor and acceptor templates were composed of RNA. Figure 2A shows the time course. The bulk of the achievable switching occurred within 1.5 h at 37°C, under the chosen reaction conditions. The extent of switching was dependent on the molar excess of acceptor template relative to donor template (Fig. 2B). The reaction was 57% efficient even when the amount of acceptor RNA was approximately equimolar with the amount of donor template. In these two series of experiments, the amount of template switching was controlled by either the reaction time or the amount of acceptor template. One interpretation is that the rate-limiting factor for switching was the probability of the elongated primary product to make an essential interaction with the acceptor template.

Figure 2C summarizes the results of examining a third variable, the extent of overlap between the donor and acceptor templates. With 100 b of overlap, there was a significant amount of template switching. And as the amount of overlap was decreased to 40 and 20 b, there was a decrease in the amount of switching. With no more than 10 b of overlap, there was no detectable switching. These data are also consistent with the above-mentioned interpretation of the rate-limiting step and indicate that the probability of the essential interaction between primary product and acceptor template is in turn dependent on the length of complementary sequence, that is, the amount of overlap between the two templates.

In the above studies, we made use of a bacterially expressed MLV reverse transcriptase, which also contains RNase H activity. In order to test the role of this RNase H activity, we made use of another recombinant enzyme. This protein lacked the carboxy terminus of the protein that contains the RNase H domain (8). As summarized in Table 1, we tested whether this enzyme could allow template switching compared with the normal reverse transcriptase. When the templates were both RNA, we observed 3% switching, indicating that the RNase H-deficient enzyme was also inefficient in switching. The measure of relative efficiency (3%) was probably an upper limit, because the



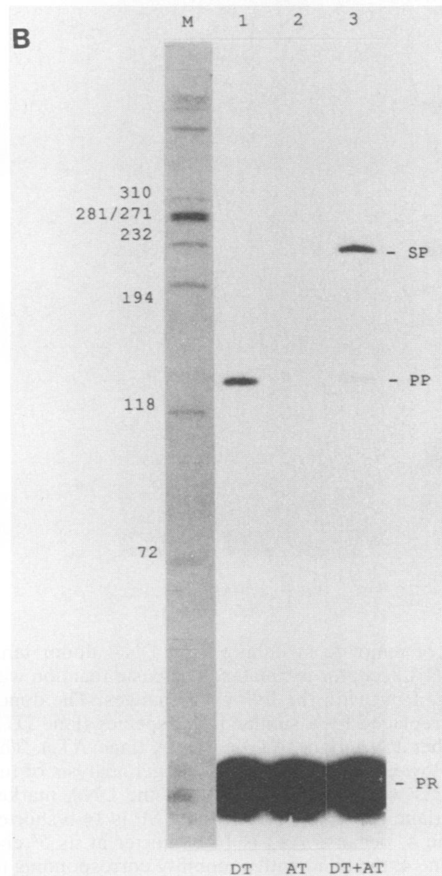


FIG. 1. Basic assay system. (A) Representation of the DNA primer, two templates, and two DNA products. The templates were composed of either RNA or DNA. The products were obtained by using reverse transcriptase. The primary product represented elongation of the labeled primer by using the donor template. The secondary product could only be generated by DNA synthesis initiated on the donor template making a switch to the acceptor template. (B) Example of analysis of the labeled DNA reaction products. PR, end labeled with ^{32}P, was incubated with donor RNA template (DT) (lane 1), AT (lane 2), and both (lane 3). DNA synthesis was carried out for 3 h at 37°C by using cloned MLV reverse transcriptase, after which the products were extracted and separated on a denaturing gel of 6% polyacrylamide. The gel was dried and analyzed by autoradiography, as shown. The free PR was separated from the primary product (PP) and the secondary product (SP). The lengths were deduced relative to the size markers in lane M, namely, end-labeled *Hae*III fragments of phage ϕX174 DNA. For quantitation of ^{32}P in the dried gel, we used an AMBIS radioanalytic imaging system. For example, in lane 3 this showed that the amount of SP relative to SP plus PP was 83%. This is referred to as the percentage of template switching.

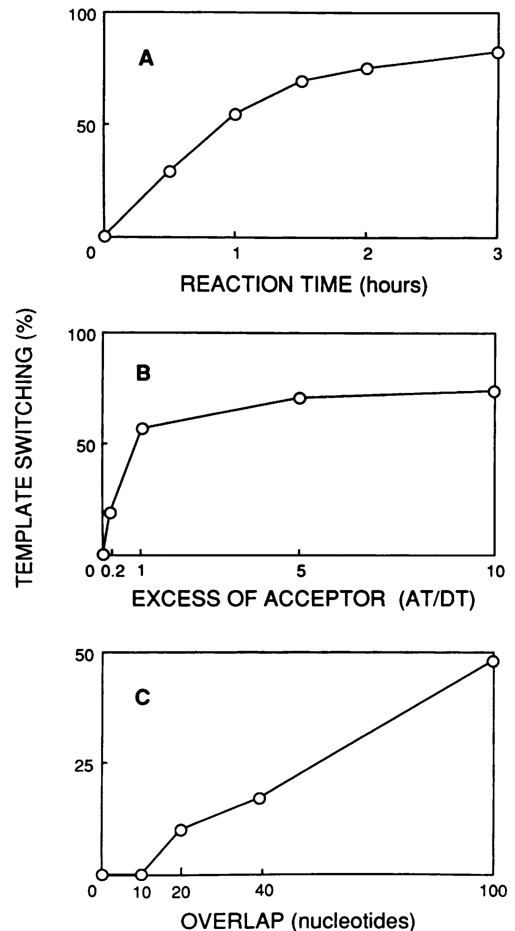


FIG. 2. Parameters affecting the extent of template switching. (A) Summary of the effect of reaction time. The reaction mixture included labeled primer, an RNA as donor template, and a fivefold excess of another RNA as acceptor template, with a 100-b overlap. The mixture included MLV reverse transcriptase and the incubation times were as indicated. Gel analysis and subsequent AMBIS scanning were used to determine the percentage of secondary product relative to the total of primary and secondary products, as described in the legend to Fig. 1. (B) Summary of reactions, as in panel A, except that the reaction times were kept constant at 3 h, while the ratio of acceptor to donor template was varied from 0 to 10. (C) Summary of reactions, as in panel B, except that the ratio of template acceptor to donor was kept constant at 5, while the lengths of the template donors were varied so that the amount of overlap with the template acceptor ranged from 0 to approximately 100 b. More precise estimates of the overlaps used are 101, 35 to 38, 20 to 23, and 3 to 8 b. These values were obtained by primer extension on the templates relative to a dideoxy sequencing ladder (15) (data not shown).

switching activity of the normal enzyme was probably saturated (Fig. 2B).

Although the deficient enzyme did not contain the coding region for RNase H, it was possible that as a consequence of the expression in bacteria, some bacterial RNase H might have been copurified with the reverse transcriptase activity. To test for this, we carried out the experiment as summarized in Fig. 3. We created an RNase H-sensitive substrate by hybridizing a DNA oligonucleotide to an end-labeled RNA (10). This substrate was incubated with either the normal or the RNase H-deficient enzyme and then tested on

TABLE 1. Requirement of RNase H domain for template switching[a]

Donor template	Acceptor template	% Template switching with RNase H: Present	Absent	Ratio of switching (absent/present)
RNA	RNA	83	3	0.03
DNA	RNA	39	ND	
DNA	DNA	11	4	0.36

[a] Template-switching experiments were carried out as described in the legends to Fig. 1B and 4; quantitation was with the AMBIS machine.

an acrylamide gel for any cleavage. The normal enzyme caused cleavage (Fig. 3, lane 2), while no detectable cleavage was observed with the deficient enzyme (lane 3).

In order to test further whether template switching was dependent on RNase H activity or not, we modified the basic assay so that the donor RNA template was replaced by DNA. Examples shown in Fig. 4 demonstrate that we were able to obtain template switching from DNA to either RNA (lane 4) or to DNA (lane 5). Table 1 gives a quantitation of such transfers, including the observation that the RNase H domain of the reverse transcriptase was not a major determinant of switching between DNA templates. (That is, the RNase H-deficient enzyme gave 36% as much switching as the normal enzyme.)

From these experiments, we concluded that reverse transcriptase could carry out significant amounts of template switching from either RNA or DNA templates and that even more efficient switching occurred when the donor template was RNA and the reverse transcriptase contained RNase H function.

We also carried out experiments to determine whether the labeled primary DNA product was actually released from the

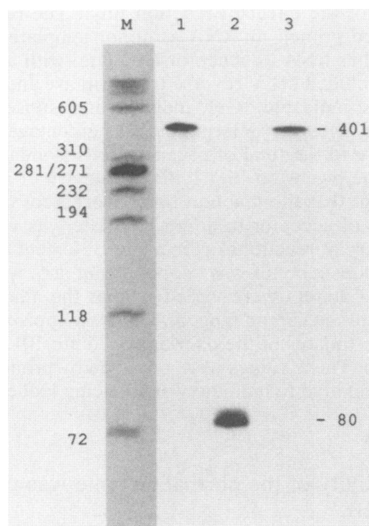


FIG. 3. Assay for RNase H activity. An RNA species, labeled at its 5′ end with [γ-^{32}P]ATP, was hybridized to a 50-b complementary DNA oligonucleotide and then used as a substrate for RNase H activity. Lane 1, No enzyme; lane 2, MLV reverse transcriptase; lane 3, MLV reverse transcriptase engineered to have none of the domain needed for RNase H function. After incubation, the samples were extracted and separated on a gel of 6% polyacrylamide, dried, and autoradiographed, as shown. The size markers in lane M are as in Fig. 1.

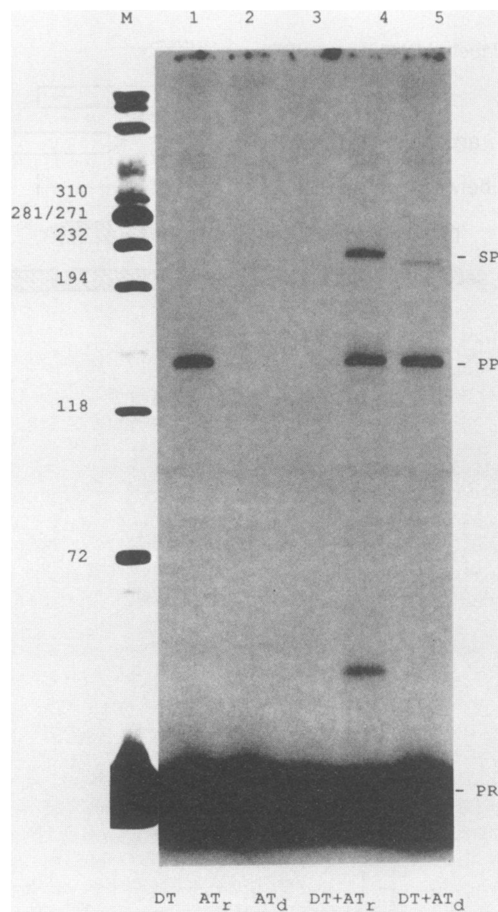


FIG. 4. Analysis of template switching from DNA donor template to RNA and DNA acceptor templates. The basic reaction was carried out as in Fig. 1 but with the following changes. The donor RNA template was replaced by a similar DNA species (lane DT), and the AT was either RNA (lane AT_r) or DNA (lane AT_d). The amount of AT_d was lower than that of AT_r. The gel analysis of the labeled DNA products was as before, including the DNA marker indicated at the left (lane M). The DNA product SP is 14 b shorter in lane 5 than in lane 4, because AT_d is 14 b shorter at its 5′ end relative to AT_r. In lane 4, the band with a mobility corresponding to about 30 b was proven to be an artifact and was solved for subsequent experiments.

donor template, even in the absence of the acceptor template. This was done by analysis of the products by using electrophoresis into nondenaturing polyacrylamide gels. As a positive control, an aliquot of each product was heat denatured prior to electrophoresis. Figure 5 shows the results obtained when the products were synthesized with the end-labeled primer (PR) and an RNA as the donor template. Figure 5, lane 1, shows the product directed by the normal reverse transcriptase. Using direct quantitation of Fig. 5, lane 1, we found that about 7% of the product was already released from its RNA template, compared with the control in lane 2. Figure 5, lane 3, shows the primary product as synthesized with the enzyme lacking RNase H function. Here, we were unable to detect any released product. Note that the complex migrated differently from that created by the complete enzyme. Our interpretation is that the complete enzyme allowed RNase H digestion of some of the RNA template that had been reverse transcribed. However, it

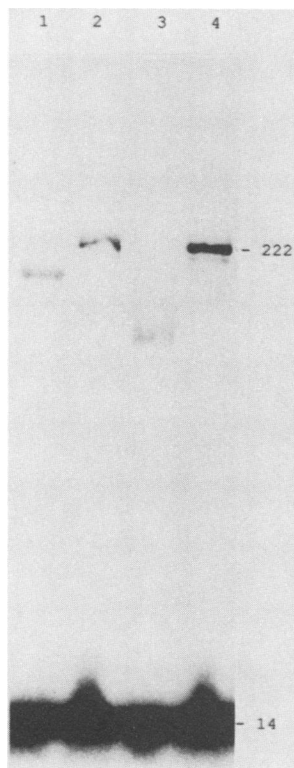


FIG. 5. Analysis of template-product associations by electrophoresis into a nondenaturing polyacrylamide gel. End-labeled primer was elongated on an RNA template by using either normal reverse transcriptase (lanes 1 and 2) or the form lacking RNase H activity (lanes 3 and 4). This template was 86 b longer than the acceptor template used in the template-switching experiments. The reaction products were directly loaded onto a nondenaturing gel of 6% polyacrylamide either without (lanes 1 and 3) or with (lanes 2 and 4) a prior heat denaturation. After electrophoresis, the gel was dried and examined by autoradiography.

must be noted that this digestion was only partial, in that it did not lead to release of the primary product.

The above experiments, with and without RNase H activity, show that after DNA synthesis, most of the product molecules are still in association with their RNA template. Similar results were obtained with a DNA template (data not shown). Our interpretation is that under the conditions of DNA synthesis used in these experiments, each template was apparently used only once. (If a template were used more than once, the synthesis of a second product, by what can be called displacement synthesis, would displace and ultimately free the first product. A theoretical argument against this occurring in our experiments with RNA templates was that in most reactions, we used a 15-fold molar excess of template relative to primer. We also carried out an experiment in which synthesis was initiated with relatively low amounts of labeled primer and then chased by the addition of an excess of unlabeled primer and more enzyme. We found that this did not lead to an increase of released labeled product [data not shown], consistent with the interpretation that displacement synthesis was not relevant to our experiments.)

From our studies with RNA donor templates (Fig. 5), we could not deduce which part(s) of the transcribed RNA was removed by the RNase H. Figure 6A diagrams five theoretical possibilities. The results shown in Fig. 5 had already eliminated the first possibility (no RNase H action) and the fifth possibility (complete RNase H action and release of free product). (The amount of free product was actually 7%, and we are attempting here to explain the remaining 93%.)

There is a dogma, arising from early studies of retroviral minus-strand strong-stop DNA (18), that RNA digestion proceeds from the 3′ end of the template in a 3′-to-5′ direction. This is diagrammed in Fig. 6A as the third possibility. In contrast, more recent studies (13) indicate that the RNase H domain may act endonucleolytically and at a distance of 7 to 14 b behind the polymerization site. This could lead to the second or fourth possibility. In order to distinguish further between the various possibilities, we carried out the following experiments.

In the first experiments, we asked whether the labeled 5′ end of the nondenatured DNA product was base paired with a residual fragment of template RNA, as in possibilities three and four. To test this, we examined accessibility of the labeled 5′-terminal phosphate in the product to digestion with calf intestinal phosphatase (CIP). Our rationale, based on the experience of others (14), was that base pairing of this region with residual RNA template would confer resistance to CIP. The products were in fact much more resistant (48%) than free DNA (6%) (Fig. 6B). However, the resistance of the product was nevertheless not as much as that of the product synthesized in the absence of RNase H (93%). (Note that all the CIP treatments [Fig. 6, lanes 2, 3, 5, and 6] successfully removed the 5′ label from the primer, PR.) Two models could be advanced to explain the intermediate level of resistance. One model is that the products are heterogeneous, with some DNAs being free, and thus CIP-sensitive, and with others still in association with the RNA template. The other model is that the products are homogeneous, but that during the CIP digestion, some of products were digested. This latter model is supported by the largely homogeneous electrophoretic migration of the nondenatured product (Fig. 5, lane 1).

A second series of experiments was then carried out to distinguish between possibilities three and four. For these, the label was not in the DNA primer but at the 5′ end of the RNA template. As in the nondenaturing gel shown in Fig. 6C, we observed the production of a labeled product complex (lane 1). This result appeared to exclude possibility three and favor possibility four. (A limitation of this experiment was that it did not exclude the possibility that some other complexes existed that were no longer associated with labeled RNA). If this interpretation were to be correct, we reasoned that it should be possible to locate the residual end-labeled RNA fragments on a denaturing gel. As shown in Fig. 6D, lane 1, this was confirmed. The fragments were predominantly of a single size. This species was 10-fold more abundant than any other. It was shown to be 17 nucleotides by using adjacent ladders of the untreated RNA after partial hydrolysis with alkali (Fig. 6D, lane OH), and partial digestion with the ribonuclease of *Phyllobacterium myrsinacearum* that is specific for the 3′ side of uridines and adenosines (Fig. 6D, lane PhyM). Actually, minor amounts of digestion occurred on some molecules at sites 1 and 2 b on either side of this site, as well as between nucleotides 9 and 10. As expected, none of these species were detected for products generated with the reverse transcriptase that lacked RNase H activity (Fig. 6D, lanes 2).

Our interpretation of these results was that some of the labeled RNA that was used as template for reverse transcriptase was cut by the associated RNase H between

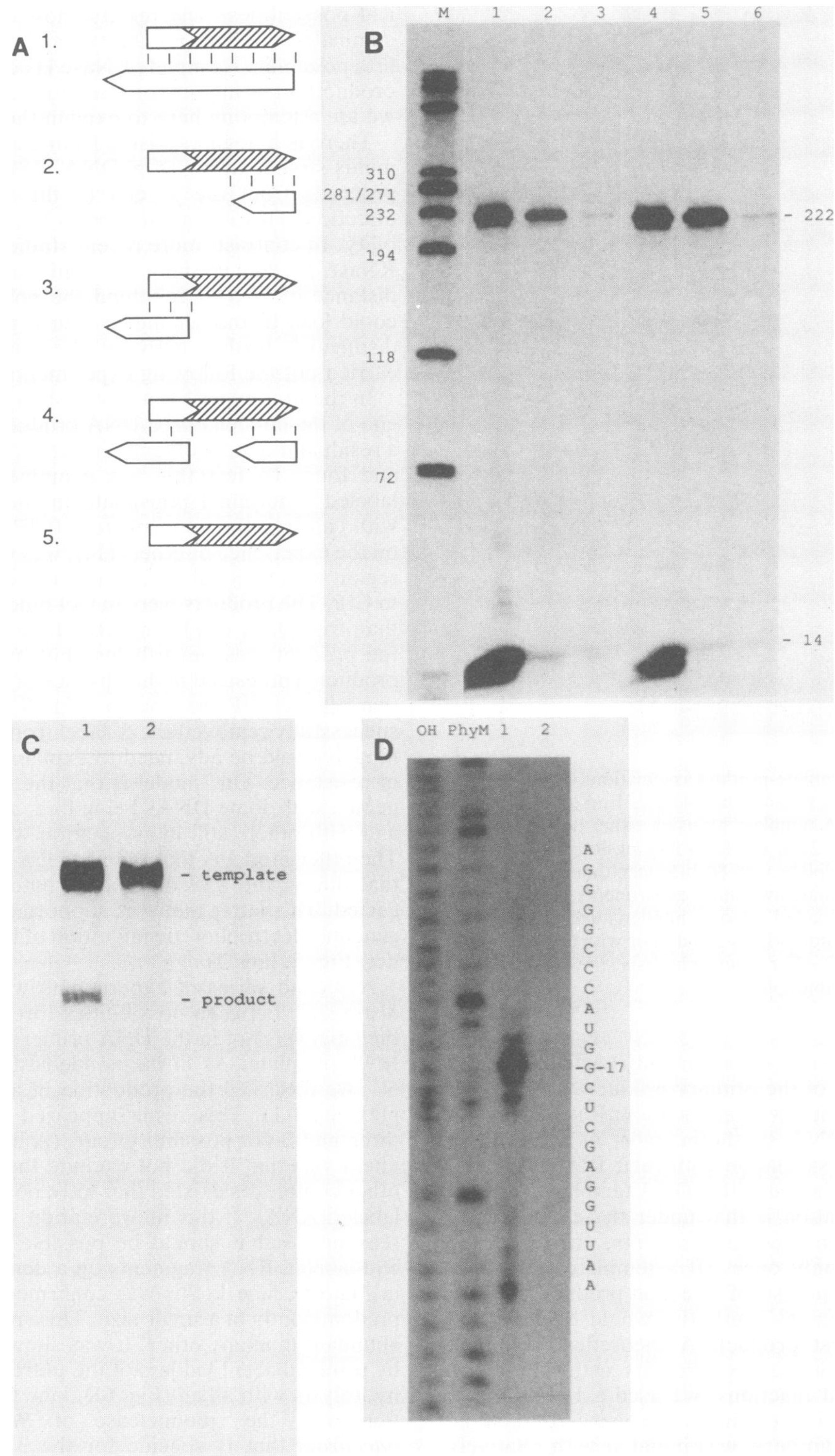


FIG. 6. Determination of structure of template-product associations. Reactions were carried out as in Fig. 5, with an oligonucleotide primer, an RNA donor template, and reverse transcriptase. (A) Five theoretical structures for the resultant template-product association, with the representations as shown in Fig. 1A and with base pairing indicated by vertical lines. (B) Denaturing gel analysis of the sensitivity of the end-labeled products to digestion with CIP. Products were generated (i) with normal reverse transcriptase (lanes 1 through 3) or (ii) with the RNase H-deficient enzyme (lanes 3 through 6). Identical portions were examined directly (lanes 1 and 4), after CIP treatment (lanes 2 and 5), or after heat denaturation and CIP treatment (lanes 3 and 6). (C) Nondenaturing gel analysis of template-product associations, as in Fig. 1B, except that the label was not on the DNA primer but at the 5′ end of the donor RNA template. Reactions were carried out with the normal reverse transcriptase. Portions were examined on a nondenaturing gel either directly (lane 1) or after heat denaturation (lane 2). (D) Sequencing gel of 10% polyacrylamide as used to examine end-labeled products from panel C. Also shown, as size and sequence markers, are a partial alkali digestion and a PhyM (Bethesda Research Laboratories) digestion of end-labeled RNA (11).

nucleotides 17 and 18, as measured from the 5′ end. This action of RNase H at a site lagging the site of DNA polymerization was exactly consistent with what has been deduced by Oyama et al. (13).

DISCUSSION

The template switching during reverse transcription, as observed in these studies, was consistent with the three basic types of switching reviewed in the introduction. Others have predicted that the various strand switches would be largely dependent on the RNase H domain of the reverse transcriptase. Our results confirm this and provide some understanding of the factors affecting template switching, especially the role of RNase H digestion. While our data support the model of recombination proposed by Coffin (1; in press), in the absence of data to the contrary, we would caution against referring to the switching as copy choice and agree with Coffin in describing the model as forced copy choice (in press). We have interpreted our data as evidence for forced switches, that is, switches forced by transcription coming to the end of a template. Actually, we have not excluded the additional possibility that some switching occurs from within the region of overlap but prior to the actual end of the donor template. We expect such switching to be relatively minor, but it could be enhanced by the presence of modified bases in the template or secondary structures in either template or nascent DNA product.

In terms of the RNase H digestion, we were able to show that after extension by reverse transcription to the end of the RNA template, the consequences of RNase H action on the transcribed RNA were as in Fig. 6A, panel 4. That is, there were residual fragments of the RNA template sufficient to make interactions with both the 5′ and 3′ ends of the DNA product. More specifically, we found that the 3′ end of the product was bound to a 17-b fragment from the 5′ end of the RNA template. These findings are consistent with the recent studies of Oyama et al. (13) and Krug and Berger (9), which show that the retroviral RNase H acts as an endonuclease. Also, the discrete size of the 5′ fragment of the template is in agreement with the concept of the reverse transcriptase as described by Oyama et al. (13); they explain the RNase H domain as acting behind the polymerase domain. That is, acting at a site between the RNA template and the newly synthesized DNA product created by one or two turns of the double helix. This residual RNA fragment created when the polymerase comes to the end of the RNA template may also be relevant to the reverse transcription of hepadnavirus genomes (12). It has been found by Lien et al. (10) that for duck hepatitis B virus, 19- and 20-b 5′ fragments of the plus-strand RNA are not only generated but go on to become primers for the reverse transcription of minus-strand DNA into plus-strand DNA. Since the 5′ end of the hepadnavirus RNA is a cap structure, reverse transcription must stop 2 b before the end of the template; that is, the RNase H acts either 17 or 18 b behind the site of termination of reverse transcription. Almost identical results have been reported by Seeger et al. (16) for two other hepadnaviruses, woodchuck hepatitis virus and ground squirrel hepatitis virus. The similarity of these results to the retrovirus data presented here is striking. It is tempting to speculate further and to suggest that as a general phenomenon, after reverse transcription to the end of an RNA template, such a residual oligonucleotide might be able to act as primer for synthesis of an almost-full-length second strand. This could have relevance not only to the technology of in vitro synthesis of double-stranded cDNAs but also to the in vivo creation of reverse transcripts of cellular RNAs as precursors for integration.

It is important to note that we have also obtained two independent lines of evidence for a mechanism of template switching that is independent of RNase H action. This second mechanism occurs at a lower but not insignificant level. Its existence has been demonstrated both by using an enzyme that lacks RNase H activity and by replacing the RNA template with a DNA template. The data support the interpretation that this additional form of template switching is not due to strand displacement, as might be expected if the template were used more than once. We do not yet understand the mechanism, but the apparent requirement for at least some unwinding of the 3′ end of the primary product is relevant not only here but for the switching mediated by RNase H actions, since we have shown that a residual RNA oligonucleotide does bind to the 3′ end, and it also has to be removed. It may be relevant that in an early report of Collett et al. (2), it was claimed that reverse transcriptase has an unwinding function. Further experiments are needed to test this hypothesis.

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