The reverse transcriptase encoded by the non-long terminal repeat retrotransposon R2 has been shown to be able to jump from the 5′-end of one RNA template (the donor) to the 3′-end of a second RNA template (the acceptor) in the absence of preexisting sequence identity between the two templates. These jumps between RNA templates have similarity to the end-to-end template jumps described for the RNA-directed RNA polymerases encoded by certain RNA viruses. Here we describe for the first time the mechanism by which such end-to-end template jumps can occur. Most template jumps by the R2 reverse transcriptase are brought about by the enzyme’s ability to add nontemplated (overhanging) nucleotides to the cDNA when it reaches the end of the donor RNA. The enzyme then anneals these overhanging nucleotides to sequences at the 3′-end of the acceptor RNA. The annealing is most efficient if it involves the terminal nucleotide(s) of the acceptor RNA but can occur to sites at least 5 nucleotides from the 3′-end. These end-to-end jumps are similar to steps proposed to be part of the integration reaction of non-long terminal repeat retrotransposons and can explain chimeric integration products derived from multiple RNA templates.

One of the most abundant classes of mobile elements found in eukaryotic genomes is non-LTR retrotransposons. These elements, also referred to as LINE-like elements or retroposons, use a relatively simple mechanism of reverse transcribing RNA templates into DNA for insertion into the genome. In the first step, the target chromosomal DNA is cleaved by an element-encoded endonuclease. The RNA-directed DNA polymerase encoded by the element then uses the free end of the cleaved target DNA as the primer to initiate reverse transcription starting at the 3′-end of the element’s RNA transcript. This process has been termed target (DNA)-primed reverse transcription (TPRT) (1). Whereas the cleavage that initiates the TPRT reaction could potentially be single-stranded (i.e. a DNA nick) or double-stranded (i.e. a DNA break), in the two systems studied in vitro the cleavage has been shown to be a DNA nick (1, 2). It has been suggested that a short region of the RNA template can anneal to the DNA immediately downstream of the nicked site, thus aiding the initiation of reverse transcription (3). However, such annealing is not required in the most extensively characterized element, the R2 element from Bombyx mori (4–6).

We are conducting a systematic study of the catalytic properties of the reverse transcriptase (RT) encoded by the R2 element (7, 8). Perhaps the most dramatic difference between R2 RT and the RT encoded by LTR retrotransposons and retroviruses is the ability of R2 RT to jump from the 5′-end of one RNA template to the 3′-end of a second RNA template. This continuous polymerization of cDNA on noncontinuous RNA templates differs dramatically from the template switching reaction associated with retroviral cDNA synthesis. Retroviral template switches occur through catalytic removal of the donor RNA from the cDNA strand by an associated RNase H domain, which then allows the cDNA to anneal to the acceptor RNA (9). The template jumps by R2 reverse transcriptase do not depend upon sequence identity between the donor and acceptor RNA and thus are similar to the template jumps that have been characterized for a number of viral RNA-directed RNA polymerases (10, 11). RNA jumps by viral RNA polymerases have been referred to as end-to-end template jumps, and we will also use that terminology here for the R2 template jumps.

A better understanding of the R2 template jumps is important. Whereas the TPRT mechanism readily accounts for the attachment of the 3′-end of the new DNA copy to genomic DNA, the means by which the 5′-end of the new copy is attached to chromosomal DNA is still cause for speculation. One model is that the polymerase, after reverse transcribing the RNA transcript, is able to jump onto the upstream DNA helix and continue DNA synthesis in a reaction similar to end-to-end template jumps (12, 13). Template jumping could also explain the lack of sequence requirements at the 5′-end of non-LTR retrotransposons revealed by their ability to integrate 5′-truncated copies into the genome. The RT can just as easily run off the end of a partially degraded RNA template as that of a full-length template. Here we show that R2 RT readily conducts template jumps from the 5′-end of the donor RNA even if they contain an inverted methyl G cap structure. We also show that nontemplated nucleotides added to the cDNA strand before the enzyme runs off the donor RNA facilitate the template jumps by annealing to the 3′-end of the acceptor RNA.

**EXPERIMENTAL PROCEDURES**

**Materials**

R2 protein was expressed in Escherichia coli JM109/pR260 and purified as previously described (7). Protein concentrations were determined on SDS-polyacrylamide gels using the fluorescent stain SYPRO Orange (Bio-Rad). The intensity of the R2 band was compared with known concentrations of bovine serum albumin using the fluorosensing function of a Storm 860 PhosphorImager and ImageQuant. Avian myeloblastosis virus (AMV) reverse transcriptase, dNTPs, and 5′-cap analog [m7G(5′ppp5′)G] was obtained from Promega. The AB.pso,
Methods

Preparations of RNA Templates—All RNA templates were generated in vitro by run-off transcription using T7 RNA polymerase (Promega). Templates for RNA synthesis were either restriction-digested pBSII(II/SK−) plasmids (Stratagene) or PCR-amplified products containing the T7 promoter. The 177-nt donor RNA was transcribed from a template generated by PCR amplification of plasmid pB108 (14) with primers AB.1 (5′-CTGCAGTAACTACCTATAGGACCTTGGAATTGC-3′) and AB.9 (5′-GATGACGAGGGCATTTGCGTA-3′). The 183-nt acceptor RNA was transcribed from a template generated by amplification of pBSH(SK−) with primers AB.8 (5′-GGAAAAGCTATGACCAGTAT-3′) and AB.T7 (5′-TAATTACGACTCACTATAG-3′). The 190-nt acceptor RNA was transcribed from a template generated by amplification of pUC18 with primers AB.22 (5′-GGAATTCGCCATTCAGGCTG-3′) and AB.15 (5′-CTGCAGTAACTACCTATAGGACCTTGGAATTGC-3′). The 334-nt vector RNA was transcribed by T7 RNA polymerase from pBSII(II/SK−) preincubated with Promega AMV reverse transcriptase and with T4 RNA ligase (Fermentas). Before ligation, the triphosphate end of the CG or AT RNA were removed by alkaline phosphatase (Promega) and repurified.

Preparation of Templates with Annealed and Cross-linked Primers—DNA templates for in vitro transcription of the AT and CG acceptor RNA were PCR-amplified using pBSH(SK−) with one primer including the T7 promoter sequence AB.T7 (5′-TAATTACGACTCACTATAG-3′) and second primer AB.CG (5′-GACAGCTATGACCATG-3′) for the CG acceptor RNA or AB.AT (5′-TTATATTCTCTAGACTTGACACTGCTG-3′) for the AT acceptor RNA, where X and Y represent C2′ methoxy-modified G and A nucleotides, respectively. PCR products, which include this C2′ methoxy-modified nucleotide at the 5′-end, were purified by gel extraction (Qiagen). All PCR reactions were performed with 20 pmol of each primer and 250 ng of pBSII(II/SK−) as described above.

End-to-end Template Jumps Are Continuous Events, Not Reinitiation Events—The template jumping ability of R2 RT can be demonstrated in any run-off reaction if an RNA acceptor was provided (7). In the assay shown in Fig. 1A, R2 RT was added to a reaction mixture containing 30 pmol of vector RNA, 0.8 pmol of acceptor RNA, and 0.8 pmol of donor RNA. The reaction mixture was incubated with 300 ng of the RNA (i.e. yields are presented relative to that determined for the zero time point) and analyzed as described above.
preincubated with a 177-nt RNA template containing a 32P-end labeled DNA primer annealed to its 3′-end. After 5 min, dNTPs and a 25-fold molar excess of a 183-nt acceptor RNA were added to start reverse transcription. The 3′-end of the acceptor RNA contained no region of sequence similarity to that of the donor RNA (see “Experimental Procedures” for the origin of these RNAs). Within 10 s, a significant fraction of the enzyme had polymerized to the end of the donor RNA, and by 20 s, a fraction of that enzyme had jumped templates and polymerized to the end of the first acceptor RNA. By 30 s, a fraction of the enzyme had made a second jump and had polymerized to the end of the second acceptor RNA template. Only small increases in cDNA products, the length of the donor RNA, or the length derived by one template jump were observed after 30 s. This is because the enzyme does not efficiently reinitiate reverse transcription on new donor RNA-primer complexes in the presence of excess acceptor RNA (data not shown).

The speed of the template jumps and the proposed trap function of the acceptor RNA suggests that R2 RT jumps to a second RNA before it dissociates from the first RNA. To test this hypothesis, the experiment in Fig. 1B was conducted. As in Fig. 1A, the donor RNA and RT were preincubated, and the reaction was started by the addition of dNTPs. In this case, however, the acceptor RNA was added at different times following the initiation of reverse transcription. The delay in the addition of acceptor RNA of only 1 min caused a dramatic drop in the level of template jumps. These assays indicate that under the conditions of our reactions, the enzyme jumps to the acceptor RNA before it dissociates from the first RNA. If no RNA acceptor is present, the enzyme dissociates from (runs off) the donor RNA. Once R2 RT has run off the donor RNA, it is not able to efficiently use the cDNA-RNA product as a primer to reinitiate reverse transcription of the acceptor RNA.

Template Jumps Occur Preferentially from the 5′-End of the Donor Template—Based on the size of the abundant cDNA products in Fig. 1 and sequence analysis of isolated products (7), most template jumps occurred between the 5′-end of the donor and 3′-end of the acceptor templates. A diffuse range of intermediate sized cDNA products that were ~7 nt longer than that produced in the absence of the AT acceptor. S.D., scan density in arbitrary units.

![Fig. 2. Physical blocks on the RNA template did not induce template jumps. A, jumping assays with and without a psoralen-cross-linked primer on the donor RNA. The 177-nt donor RNA template was annealed to one primer near its 3′-end to initiate reverse transcription and a psoralen-modified oligonucleotide near its 5′-end. In lanes 1–3, the psoralen nucleotide was cross-linked to the donor RNA by UV radiation. In lanes 4–6, no UV cross-linking was conducted. Lanes 1 and 4, RT reaction without acceptor RNA; lanes 2 and 5, reaction with a 183-nt acceptor RNA; lanes 3 and 6, reaction with a 332-nt acceptor RNA. B, gel scans of lanes 2 and 4 from the gel shown in A. The covalent bound primer blocks polymerization 50 nt from the 5′-end of donor template. Shown at the bottom are the expanded and highly overexposed regions of the gel that would contain the jumps that might arise from the psoralen cross-linked internal site. No cDNA products are seen that are ~50 nt shorter than the end-to-end jumps. S.D., scan density in arbitrary units.](http://www.jbc.org/)

![Fig. 3. Pauses in reverse transcription did not induce template jumps. A, schematic diagram of the pause-driven assay. RNA templates are shown in gray, whereas the DNA primer and cDNA are shown in black. Reverse transcription is initiated on the donor RNA template, but only four nucleotides can be incorporated because the assay contains only TTP and dATP. The acceptor RNA (AT acceptor) contains a series of seven A and T nucleotides at its 3′-end. If internal jumps from the donor RNA to the end of the acceptor RNA are possible, then the cDNA can be extended another 7 nucleotides. B, PhosphoImager scans of the pause-driven template jumping assay. Gray scan, RT reaction without the AT acceptor RNA; black scan, RT reaction with the AT acceptor RNA. The addition of the AT acceptor RNA did not induce the formation of cDNA products that were ~7 nt longer than that produced in the absence of the AT acceptor. S.D., scan density in arbitrary units.](http://www.jbc.org/)
assays (8). We have therefore attempted several methods to block polymerization to determine whether a stalled enzyme could be induced to jump between RNA templates. However, neither hairpin structures in the donor RNA nor long oligonucleotides annealed to the donor RNA induced R2 RT to pause during polymerization (8). We therefore generated a covalent block on the RNA template. The blockage was generated by psoralen cross-linking an annealed oligonucleotide to the RNA donor 52 nucleotides from its 5′-end. The efficiency of the cross-link was about 20%. As shown in Fig. 2, psoralen cross-linking catalyzed by UV irradiation induced the formation of a cDNA product that was ~52 nt shorter than the full-length product (comparison of lanes 1 and 4). In these lanes, extension products larger than the 177-nt cDNA were not generated, because the saturating levels of the annealed primer effectively blocked the RT from template jumps onto another donor RNA.

In lanes 2 and 5, the 183-nt acceptor RNA was added to the reverse transcription reaction, and in lanes 3 and 6, a different (332-nt) RNA acceptor was added to the reaction. If template jumps from the internal blocked site on the donor had occurred, the jumping products would be about 50 nucleotides shorter than that in the absence of the cross-link. Shorter products than the normal end-to-end jumps were not detected even with extensive overexposure of the appropriate region of the gel (Fig. 2B), suggesting that blocked R2 RT seldom undergo internal template jumps.

Additional evidence that R2 RT template jumps could only occur from the 5′-end of the donor RNA came from experiments in which enzyme pausing within the donor RNA was induced by nucleotide depletion. As diagrammed in Fig. 3A, an RNA extension reaction was conducted in the absence of dCTP and dGTP. Under this condition, the polymerase can incorporate four A and T nucleotides before reaching four contiguous G and C residues. As shown by the gel scans in Fig. 3B, the R2 enzyme was efficiently blocked by this approach with about one-half of the cDNA extension products ending at the first G residue of the template and the other half of the cDNA containing one misincorporated nucleotide. To determine whether template jumps could be induced by this pause in the polymerization, an acceptor RNA was added to the reaction that contained seven A and T nucleotides at its 3′-end. If R2 RT was able to jump to the 3′-end of this acceptor RNA, it would incorporate another 7 nucleotides before again being forced to stop at a sequence of adjacent G and C residues. As shown by the second cDNA profile scan in Fig. 3B, no increase in cDNA products ~7 nt longer than the original blockages were detected. Because the template jumping efficiency can be affected by the dNTPs concentration (see below), a series of similar experiments with different concentrations of dATP and TTP (2.5–250 μM) was conducted. No concentration of nucleotides generated observable jumping products (data not shown). We suggest that R2 RT is extremely inefficient at template jumps from internal positions of the donor RNA.

**Effects of the Structure of the 5′-End of the RNA Donor—End-to-end template jumps require that the 3′-end of the acceptor RNA be positioned near the 5′-end of the donor template and in the proper orientation to the active center of the polymerase. Bulky structures at the 5′-end of the donor template might therefore interfere with such positioning. We tested the efficiency of template jumps from RNA donor templates that contained three types of 5′ ends: a triphosphate generated during typical in vitro transcription reactions, a cap analog (m7G-(5′ppp(5′))G), and a 5′-hydroxyl generated by dephosphorylation of the RNA donor. The results of these experiments are shown in Fig. 4. Up to five template jumps can be seen for all templates. It is important to note that only the first template

![Fig. 4. Effects of the donor template 5′-end on the efficiency of the template jumps. Lane 1, the donor RNA was synthesized with the cap analog, m7G(5′ppp(5′))G; lane 2, donor RNA from a standard T7 reaction was treated with alkaline phosphatase to leave only a 5′-hydroxyl (OH); lane 3, the donor RNA was used directly after standard T7 transcription and thus ends in a 5′-triphosphate (PPP). The yield of end-to-end jumps was calculated as the combined total of all jump products (up to five consecutive jumps can be seen) divided by the total level of cDNA products that are greater than or equal to full-length donor products.](http://www.jbc.org/)

![Fig. 5. Effects of the donor template 5′-end on the efficiency of nontemplated nucleotide incorporation. Run-off assays (i.e. no acceptor RNA) were conducted using the 177-nt donor RNAs with the 5′ structures described in the legend to Fig. 4. Upper panel, autoradiograph of the 177-nt region of a high resolution polyacrylamide gel. Lanes 1, 3, and 5, R2 RT; lanes 2, 4, and 6, AMV RT. Lanes 1 and 2, 5′-cap analog; lanes 3 and 4, 5′-triphosphate; lanes 5 and 6, 5′-hydroxyl. Lower panel, PhosphorImager scan of the R2 assays shown in the upper panel. Position 0, the length of cDNA expected if it terminated at the 5′-nucleotide of the donor RNA (177 nt); positions 1–3, the addition of additional (nontemplated) nucleotides to the end of the cDNA.)
jump was from the 5'-modified donor 177 RNA. All subsequent jumps were between the 183-nt acceptor RNAs, which contain a triphosphate 5'-end. The yield of template jumps decreased with increasing size of the 5'-overhanging group: 65% of the cDNA reactions underwent a template jump with the 5'-hydroxyl group (lane 2), 44% for the terminal triphosphate (lane 3), and 34% for the 5'-cap structure (lane 1). Thus, the efficiency of the end-to-end template jump decreases with the presence of more bulky structures at the 5'-end of the donor RNA. However, even in the case of the inverted G residue of a 5'-cap, the inhibition was only 2-fold.

The Incorporation of Additional (Nontemplated) Nucleotides during the Template Jumps—Small differences in the length of the cDNA products not undergoing template jumps can be distinguished in Fig. 4. Higher resolution polyacrylamide gel analysis of these “run-off” cDNA products are shown in Fig. 5A (lanes 1, 3, and 5). Shown below this gel are scans of the R2 RT run-off products with position 0 referring to the last position of the RNA template and positions 1–3 referring to the presence of additional (overhanging) nucleotides at the end of the cDNA. Also presented in this figure are run-off products using the RT of the AMV (lanes 2, 4, and 6). AMV RT produced cDNA the exact length of the RNA template when that template contained either a 5'-hydroxyl group or 5'-triphosphate. Full-length cDNA products and products 1 base pair shorter than full-length were generated by AMV RT on the 5'-capped RNA. In contrast to AMV RT, the run-off products for R2 RT were longer and more dependent upon the 5'-end of the RNA template. In the case of the RNA with the 5'-hydroxyl, the major product was cDNA that contained three extra nucleotides. For the RNA with the 5'-triphosphate, the cDNA products were nearly equally distributed between those containing 1, 2, or 3 extra nucleotides. Finally, for the 5'-capped RNA, the major cDNA product was the precise length of

Fig. 6. Structure of the donor/acceptor junctions after R2 template jumps. A, junctions generated by direct ligation of the donor and acceptor RNA. Donor and acceptor RNAs were ligated by T4 RNA ligase, the junctions were RT-PCR-amplified, and the products were digested with SpeI before separation on a high resolution polyacrylamide gel. Top panel, PhosphorImager scan of the ligated products when the acceptor RNA was generated from a C2'-methoxy-modified DNA template to prevent nontemplated additions by T7 RNA polymerase. Lower panel, ligated products when the acceptor RNA was synthesized off a standard DNA template. Position 0, perfect junction (direct ligation of the last nucleotide of each RNA based on the DNA templates used to make the RNA). Positions 1–3, junctions contain extra nucleotides due to nontemplated additions by the T7 polymerase; positions -1 to -2, junctions shorter than expected. B, analysis of the donor/acceptor junctions generated by R2 RT. Upper panel, autoradiogram of the resolved products generated from donor RNA templates containing the different 5' junction described in the legend to Fig. 4. Lower panel, PhosphorImager scan of the high resolution gel. Positions -2 to 1 are the same as described in A. C, summary of the direct sequencing of the donor/acceptor junctions using the triphosphate donor RNA (PPP). Because the junction has both additional and missing bases, one cannot “read” the DNA sequence beyond the junction. Therefore, two sequencing reactions were conducted, one from within the donor 177 nt RNA (top) and one from within the AT acceptor RNA (bottom). In both cases, the RNA sequence lies within a shaded box, and the sequence read from the gel is shown below. A unique nucleotide indicates that base was present in over 80% of the amplified product. Near the end of the acceptor RNA and beyond the end of the donor, the sequences became ambiguous (2 or 3 nucleotides at each position), or there was no clear preference (scored as N).
the RNA template, with the remaining cDNA products containing one or more extra nucleotides. The run-off products are similar whether or not an acceptor RNA is present in the reaction (compare Figs. 4 and 5). In addition, run-off assays conducted in the presence of heparin, which blocks R2 reinitiation (8), resulted in the same extension profiles (data not shown). Thus, the addition of extra nucleotides by R2 RT occurs before the enzyme falls off of the RNA template and not after reinitiation and extension of the cDNA. The ability to add extra nucleotides when a polymerase reaches the end of its polynucleotide substrate has been observed with other nucleic acid polymerases (16–21), but it is unusual for so many nucleotides to be added so efficiently (see “Discussion”).

We next determined whether extra nucleotides were incorporated during the end-to-end template jumps catalyzed by R2 RT. Additional nucleotides at the junction of the donor and acceptor RNA could be a result of the extra nucleotides added by R2 RT during the jump or by extra nucleotides added to the 3’-end of the acceptor RNA during its synthesis by T7 RNA polymerase (21). To eliminate the extra nucleotides added by T7 RNA polymerase, we used a modification of the method of Kao et al. (15). In this method, the penultimate nucleotide of the DNA templates used for T7 transcription was each methoxy-modified template were each ligated to the donor RNA (see “Experimental Procedures”). The products of the ligation were then amplified by RT PCR and digested with a restriction enzyme that cleaved near the jump site, and the products were separated on a polyacrylamide gel (Fig. 6A). A large fraction of the RNA generated by a standard T7 transcription reaction contained 1 or 2 extra nucleotides. (Position 0 on this scan represents the length expected for the ligation of the last nucleotide of the donor RNA to the first nucleotide of the predicted RNA acceptor.) In contrast to the RNA generated by standard means, the majority of the ligated RNA products synthesized off the C2’ methoxy-modified templates contained no extra nucleotides.

**Table 1**

| dNTP Concentration | AT acceptor | CG acceptor |
|--------------------|-------------|-------------|
| 2.5 μM dNTPs       | 12%         | 23%         |
| 2.5 μM dNTPs + 500 μM dGTP | 9%      | 63%         |
| 2.5 μM dNTPs + 500 μM dATP | 41%     | 21%         |
| 2.5 μM dNTPs + 500 μM dCTP | 17%     | 42%         |

**Fig. 8.** Effect of the dNTP concentration on the efficiency of nontemplated nucleotide incorporation. A, shown are the scans of the 177-nt region of a high resolution gels. The numbers at the right of each graph represent concentration of all four dNTPs. Position 0 represents the cDNA length if it terminated at the 5’-nucleotide of the donor RNA (177 nt); positions 1–3 represent the addition of additional (nontemplated) nucleotides to the end of the cDNA. B, effect of a higher concentration of one dNTP on the efficiency and characteristics of nontemplated additions. In each panel, the concentration of three dNTPs was 2.5 μM, whereas the concentration of the dNTP indicated was 500 μM.
Using this acceptor RNA with the more uniform 3′ termini, we then tested the junctions generated by R2 RT template jumping from donor RNAs containing a 5′-end triphosphate, the cap analog, or a 5′-hydroxyl. As shown in Fig. 6B, the junctions generated by template jumping were variable in length, ranging from 1 nucleotide longer to 2 nucleotides shorter than the combined lengths of the two RNAs. Junctions generated by template jumping from the RNA donor with the triphosphate and 5′-hydroxyl ends were predominantly of length 0 or 1 or 2 nucleotides shorter. In the case of jumps from the 5′-capped RNA, the shorter junctions were reduced, and there were larger numbers of junctions of length +1.

The significant number of junction that were shorter than the length of the ligated RNA products could be a result of the RT not transcribing to the end of the donor RNA before jumping or jumps to internal sites of the acceptor RNA. To resolve this issue, the products of the template jumping reaction from the RNA donor with terminal 5′-triphosphate were PCR-amplified, and the heterogeneous junctions were directly sequenced. The sequencing was conducted starting from both the donor and acceptor sides of the hybrid sequence. The results of these sequencing reactions are summarized in Fig. 6C. The sequence initiated from the donor template side of the junction revealed a uniform sequence extending to the end of the donor RNA, followed by a position with an A or G nucleotide. After this point, each position showed mixed nucleotide signals. Thus, a majority of the polymerase reverse transcribes to the 5′-end of the donor RNA. The sequencing initiated from the acceptor template side of the junction revealed a uniform sequence extending to the third nucleotide from the 3′-end of the acceptor (defined in Fig. 6C as position −2). The second nucleotide from the 3′-end acceptor (position −1) was composed of predominately C, A, and T nucleotides. Based on these sequences and the size of the cDNA products (Fig. 6B), most template jumps occur from the end of the donor RNA, and reverse transcription resumes at one of the first three nucleotides at the 3′-end of the acceptor RNA.

Factors Affecting the Template Jumping Reaction—To better understand the mechanism of template jumping, acceptor RNAs were tested that contained different 3′-end sequences. One of the sequences contained seven A and T residues at its 3′-end and will henceforth be referred to as the AT acceptor RNA (this RNA was used in Fig. 4). The second RNA contained four G and C residues at its 3′-end and will be referred to as the CG acceptor RNA. Both acceptor RNAs were synthesized from 2′-methoxyl-substituted DNA templates to generate homogeneous 3′-ends. Jumping reactions were conducted at various concentrations of dNTP (0.25–250 μM). As shown in Fig. 7A, a 1000-fold increase in dNTP concentration greatly stimulated the yield of end-to-end template jumps to both acceptors.

Comparisons of the lengths of the junctions produced by the template jumps (Fig. 7B) also demonstrated significant differences in the distribution of junction lengths. In general, the jumps to the AT acceptor RNA were more precise especially at lower dNTP concentrations. As the nucleotide concentration was raised, more junctions were formed that were 1–2 nucleotides shorter. The jumps to the CG acceptor were more variable, with jumps up to 4 nucleotides shorter than full-length. Also in contrast to the AT acceptor, jumps to the CG acceptor became more precise at higher dNTP concentrations.

Whereas these differences could suggest that R2 RT had different abilities to bind acceptor RNA with GC or AT 3′-ends, further experiments indicated that these differences reflected the ability of the enzyme to add nontemplated nucleotides to the cDNA before the jump. As shown in the run-off assays in Fig. 8A, as the concentration of nucleotides was increased from 0.25 to 250 μM, the fraction of the run-off products with additional nucleotides increased from 10% of the products to 97%. The number of added nucleotides was also dramatically changed from typically 1 nt at the lowest concentration to 2–4 nucleotides at the highest concentration. To examine whether the RT had a preference for adding specific nucleotides at the end of the donor RNA, separate run-off assays were conducted with a uniform low level of three dNTPs (2.5 μM) and a 200-fold excess of 1 nucleotide (500 μM) (Fig. 8B). The RT clearly showed a preference for which nucleotides were added in the run-off reaction. The cDNA products were extended 1 extra nt in the case of excess dTTP, 1–2 nt for extra dCTP, 2 nt for dGTP, and finally 3–4 nt for dATP. Thus, R2 RT prefers to add extra purine residues, particularly A residues, similar to what has been characterized for other polymerases (16–20).

We next assayed template jumps to both the AT and CG acceptor RNAs in the presence of the excess individual dNTPs. The efficiencies of the template jumps are summarized in Table I, whereas the lengths of the donor/acceptor junctions are shown in Fig. 9. High concentrations of dATP in the assay significantly inhibited the jumps to both the AT and CG acceptor RNAs (Table I). Presumably, the cDNA overextended by 3–4 A residues under these conditions (Fig. 8) were poor substrates for template jumps. The presence of extra dGTP in the
Fig. 10. Proposed mechanism of end-to-end template jumping by R2 RT. Donor and acceptor RNAs are indicated by the gray bars, cDNA is shown by the striped black bar, and R2 RT is shown by the gray oval. The thickness of the arrows indicates the probability of each step. Step A, R2 RT upon reaching the 5’-end of the donor RNA readily adds nontemplated nucleotides, usually A or G. R2 RT is able to bind another RNA template (acceptor) and anneal the overhanging nucleotides of the cDNA to positions at or near the 3’-end of the acceptor RNA (step B). A low level of template jumps without the aid of overhanging nucleotides may occur (step C), but this is an inefficient reaction compared with step B. If no acceptor RNA is bound by R2 RT, additional nontemplated nucleotides are added to the cDNA strand (step D). The relative rates of steps B versus D depend upon the concentration of nucleotides and acceptor RNA and the sequence at the 3’-end of the acceptor RNA. If 3 or 4 nontemplated nucleotides are added, then R2 RT rapidly dissociates from the donor RNA-cDNA complex (step E).

assay inhibited template jumps to the AT acceptor but increased the jumps to the CG acceptor to a level of 63% (Table I). Analysis of the nature of these junctions indicated that a high percentage of the jumps were precise (Fig. 9B), suggesting that the extra 1–2 G residues on the overextended cDNA were able to anneal to the terminal C residues of the acceptor RNA and thus increased the efficiency of the template jump.

In a similar manner, the presence of excess dTTP, which would give rise to cDNA products overextended by 1 T nucleotide, stimulated jumps to the AT acceptor RNA (Table I). These template jumps were again precise (Fig. 9). It is interesting to note that whereas this extra T on the cDNA did not change the efficiency of the jumps to the CG acceptor RNA, it did have a significant affect on the location of these jumps. Most of the jumps to the GC acceptor occurred to the third base (position –2 in Fig. 9B). This position of the CG acceptor corresponds to a G and thus would be able to anneal to the terminal T residue of an overextended cDNA. Finally, overextension of the cDNA by 1–2 C residues (Fig. 7) had little effect on the efficiency of the jumps to the AT acceptor and gave rise to a broad range of junctions. In the case of the CG acceptor, the efficiency of the jumps was increased to 43%, and many of the jumps were to the internal G residue.

These results suggest that R2 RT uses nontemplated (overhanging) nucleotides at the termini of the cDNA to anneal to nucleotides near the 3’-end of the acceptor. The annealing of overhanging nucleotides is most efficient if it involves the 3’ terminal nucleotide of the acceptor RNA, but such annealing can occur to any nucleotides within the first 5 positions of the 3’-end of the acceptor RNA.

**DISCUSSION**

End-to-end template jumping is an unusual mechanism of template recruitment that allows R2 RT to generate a continuous cDNA product derived from two or more RNA templates. In this report, we have focused on various aspects of the mechanism of this reaction including the location of the jumping events, the role of the acceptor template sequence in the vicinity of the jump, and the role played by the incorporation of nontemplated nucleotides. R2 template jumping differs from the template switching mediated by retroviral RT, because it does not depend upon preexisting sequence identity between the donor and acceptor templates, RNase H activity, or secondary structure transitions (22–25). We propose that in the R2 template jumping reaction, the donor and acceptor RNA templates have mainly a passive function. It is the R2 RT that binds the acceptor template and positions the 3’-end of the acceptor template in proximity to the 5’-end of the donor template. Because the R2 enzyme stabilizes the binding of these two templates, extensive nucleotide identity is not required.

Fig. 10 summarizes the two competing reactions that are possible when R2 RT reaches the end of an RNA template. The first reaction is the addition of nontemplated nucleotides (step A). Whereas all four nucleotides can be used in this reaction, the R2 enzyme prefers to add nontemplated purines, particularly A. Many polymerases have been shown to add an extra nucleotide in such run-off reactions (17–21), but the R2 enzyme is unusual in that under standard assay conditions and nucleotide concentrations (25–250 μM dNTPs) and in the absence of template jumps, over 90% of the cDNA contains an average of three extra nucleotides.

Competing with the addition of nontemplated nucleotides is the ability of the enzyme to bind the 3’-end of an acceptor RNA. Efficient template jumps occur if one or more overhanging nucleotides on the cDNA are able to anneal to the 3’-end of an acceptor RNA (step B). Very little template jumping appears to occur in the absence of overhanging nucleotides on the cDNA (step C). As summarized in Table I, extra G residues at the end of the cDNA significantly increased the efficiency of the jumps to an acceptor RNA that ended in C residues but inhibited the jumps to an acceptor RNA ending in A residues. In a similar manner, extra T residues on the cDNA increased the efficiency of the jumps to the acceptor RNA ending in A residues but not the jumps to the acceptor RNA with terminal C residues. Whereas annealing of the overhanging nucleotides on the cDNA to the terminal nucleotides of the acceptor RNA is most efficient at promoting template jumps, annealing can readily
occurs to residues near the 3′-end of the RNA. For example, the presence of extra C or T residues on the cDNA promoted jumps to the G residue 3 nucleotides from the 3′-end of the GC acceptor RNA (Fig. 9). In most instances, failure of R2 RT to jump was a result of the enzyme’s inability to find the 3′-end of an acceptor RNA that could anneal to the overhanging nucleotides (step D). After the addition of multiple nontemplated nucleotides, the enzyme dissociates from the donor RNA-cDNA complex (step E).

The balance between these two competing reactions, nontemplated addition and template jumps, is therefore determined by the concentration of each nucleotide, the concentration of the acceptor RNA, and the sequences at the 3′-end of the RNA. In most of our reactions, the kinetics of the positioning of the 3′-end of the acceptor RNA was in the same range as the kinetics of nontemplated nucleotide addition. If this were not true, a high percentage of the jumps would have extra nucleotides. Only in the case of high levels of dATP did the kinetics of nontemplated additions outpace that of acceptor binding. In high concentrations of dATP, jumps to the AT acceptor RNA were inefficient, although this RNA contained T residues at the second and third positions from the 3′-end. This finding suggests that once the R2 enzyme has added 3–4 nontemplated residues, it rapidly dissociates from the donor RNA. Based on these findings, it should be possible to promote the direction and efficiency of template jumps from any RNA by adding higher concentrations of dGTP to a standard reverse transcription reaction and adding an acceptor RNA that ends in a short run of Cs.

End-to-end template jumping has been previously reported for a number of nucleic acid polymerases. RNA-directed RNA polymerases from plant and animal RNA viruses have been shown to undergo end-to-end template jumps (10, 11, 26, 27). These jumps have been postulated to provide an advantage to the virus by enabling recombination between and within viruses. Whereas the mechanism of these template jumps has not been described, it is interesting to note that the most efficient end-to-end jumping was by the enzyme from the bovine viral diarrhea virus (11), the enzyme that also had the greatest affinity to add extra nontemplated nucleotides in run-off assays (20). The bovine viral diarrhea virus enzyme has also been suggested to be able to jump templates from internal positions of the donor RNA (11). We have not been able to demonstrate this activity with R2 RT (Figs. 2 and 3).

End-to-end template jump assays for L1 and TRAS (31, 32) or of integrated chimeric non-LTR retrotransposons.2 The eventual goal is to observe efficient template jumps to the target DNA during our TRP assay and thus move us one step closer to a complete in vitro retrotransposition reaction.

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