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Selective cleavage in the avian retroviral long terminal repeat sequence by the endonuclease associated with the αβ form of avian reverse transcriptase

(reverse-transcriptase endonuclease/site-specific cleavage/integration)

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ABSTRACT **M13 recombinant DNA clones containing a 350-base sequence derived from the *Eco*RI fragment of two tandemly linked Rous-associated virus 2 (RAV-2) long terminal repeat (LTR) sequences have been used to map reverse transcriptase-associated endonuclease (RT-endonuclease) cleavage sites by primer extension studies. Under appropriate conditions, the αβ form of RT-endonuclease (composed of both the α and β subunits) purified from Avian sarcoma virus (Pr-C and B-77 strains) introduces a specific break in the inverted complementary repeat sequence found at the junction of the LTRs. The cleavage sites occur in the same nucleotide sequence in (−) and (+) DNA strands; together they have the potential of generating a 6-base-pair staggered overlap that spans the junction. This supports the notion that the enzyme is involved in viral DNA integration. Other RT-endonuclease sites were analyzed. A second site, which occurs in the *lac* region of the M13 vector DNA upstream from the unique *Eco*RI cloning site, bears no apparent sequence homology to the site at the junction of the LTRs. However, it also lies within an inverted complementary repeat and, as is the case for the site in the LTR, the break occurs to the 5′ side of the axis of symmetry. Cleavage at this second site is suppressed when the vector contains the RAV-2 LTR insert. Thus, the viral LTR appears to exert a *cis* effect that can influence a region over 300 base pairs away.**

The avian retrovirus reverse transcriptase has a DNA endonuclease activity that has been proposed to function during the integration of viral DNA into the host cell's genome (1). The predominant form of the enzyme isolated from purified viral particles contains two polypeptide chains, referred to as the α and β subunits, which have molecular masses of 68,000 and 91,000 daltons, respectively (2, 3). The α subunit is formed from the β subunit by proteolytic cleavage, which releases a 32,000-dalton protein, pp32 (2, 3). Endonuclease activity is associated with the αβ and ββ forms of the enzyme and with the pp32 protein but not with the α subunit (1, 4). This suggests that the active site for the endonuclease activity is in the carboxyl-terminal domain of the β subunits as defined by pp32. The general properties of this endonuclease have been characterized (5). It requires the presence of divalent cations: Mg^{2+} or Mn^{2+} is used by the ββ form and by the pp32 protein, and Mn^{2+} is used by the αβ form. The enzyme introduces both single- and double-strand breaks with 3′ OH and 5′ P termini. Preference for substrate appears to be based upon the availability of single-stranded regions in DNA. Recently, using DNA DNase I-protection techniques, Misra *et al.* (6) have shown that the pp32 protein binds preferentially to viral long terminal repeat (LTR) sequences in recombinant molecules and other promoter-containing regions in the prokaryotic vector.

In earlier studies (5) we used clones derived from unintegrated replicative form (RF) I retroviral DNA molecules to test for specificity of the endonuclease reaction. The clones contained the LTR sequences that are found at the termini of integrated proviruses and are presumed to contain sites recognized by the viral integration machinery. Analysis of the rates of cleavage failed to reveal any preference for LTR-containing recombinants. Other experiments with restriction endonuclease analyses indicated that there were several potential sites for cleavage in both vectors and recombinants. Thus, if the endonuclease is involved in integration, some feature of the substrates' structure inside the cell must restrict the reaction to the LTR region, perhaps by means of preferential denaturation. In the study reported here, we used single-stranded substrates and mapped αβ cleavage sites in the LTR region by the use of primer extension methodology. This assay allows us to search for specific cleavages in selected regions of the DNA substrates. Our results show that, under defined conditions, RT-endonuclease can introduce specific breaks in the LTR sequences. These breaks are near the junction of tandemly linked LTRs and together could potentially generate a staggered cut spanning the LTR junction.

MATERIALS AND METHODS

Materials. [α-^{32}P]dCTP (400–600 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. Unlabeled deoxy- and dideoxynucleotide triphosphates were obtained from P-L Biochemicals. The M13 primer pentadecamer and the M13 hybridization probe primer were obtained from New England BioLabs. Pancreatic DNase was from Worthington. *Escherichia coli* DNA polymerase I (Pol I; large Klenow fragment) was from Boehringer Mannheim and New England BioLabs. M13 subclones were prepared previously from segments of Rous-associated virus 2 (RAV-2) retroviral DNA. The designation RAV2-2 identifies the particular RAV-2 DNA clone from which the M13 recombinants were derived (7).

Endonuclease Assay Conditions. The αβ and the ββ forms of reverse transcriptase were purified from avian sarcoma virus (ASV) (Pr-C and B-77) particles (5). The endonuclease was assayed in a final volume of 10 μl in a 1.5-ml Eppendorf centrifuge tube and contained 20 mM Tris·HCl (pH 8.5), 5 mM 2-

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Abbreviations: RT-endonuclease, reverse transcriptase-associated endonuclease; LTR, long terminal repeat; RF, replicative form; RAV-2, Rous-associated virus 2; ASV, avian sarcoma virus; Pol I, DNA polymerase I.

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mercaptoethanol, 500 ng of single-stranded circular M13mp2 RAV2-2 (+/−) phage DNA, as indicated, and 10 polymerizing units of reverse transcriptase (5). The mixture was incubated for 2 min at 24°C, and then 2 μl of either 6 mM $MgCl_2$ (ββ-form reaction) or 6 mM $MnCl_2$ (αβ-form reaction) was added to the incubation, bringing the volume to 12 μl. After incubation at 24°C for lengths of time as indicated, the reaction was stopped by heating to 65°C for 5 min and then placing the samples in ice. (In control experiments, the RT-endonuclease was removed from the reaction at this step by phenol extraction. Because this did not alter the results of analyses described below, the simpler heat-inactivation procedure was used in our routine assay.) A 2-μl aliquot from each reaction mixture was removed and analyzed by agarose gel electrophoresis (5) to determine the extent of cleavage of the DNA. The remainder was used to determine the sites of cleavage as described below.

Preparations and Analysis of Primer-Extended Products. The endonuclease cleavage sites in the treated DNA were determined by taking the remaining 10 μl of the above reaction mixture and adding either 0.5 ng of M13 primer pentadecamer or 0.5 ng of M13 hybridization probe primer, 1.2 μl of 70 mM Tris·HCl (pH 7.5), 70 mM $MgCl_2$, and 500 mM NaCl. The mixture was brought to a final volume of 15 μl, heated to 90°C for 3 min, and slowly cooled to 37°C. An 18.5-μl sample containing 13.3 μCi of [α-^{32}P]dCTP, 1.33 units of Pol I (Klenow fragment), 0.13 μmol of dithioerythritol, 1.0 nmol each of TTP, dATP, and dGTP, 0.5 μmol of $MgCl_2$, 0.5 μmol of Tris·HCl (pH 7.5), and 3.5 μmol of NaCl was added to the hybridization mixture. The reaction mixture was then incubated at 30°C for 15 min, at which time the four deoxynucleotides at 3.5 μmol each were added to a final volume of 40 μl. The sample was incubated for an additional 15 min at 37°C. The reaction was stopped and analyzed by gel electrophoresis as described by Maxam and Gilbert (8). The nucleotide sequence of the RT-endonuclease cleavage sites was inferred from side-by-side comparison with a dideoxy sequence ladder on DNA not treated with RT-endonuclease (9). The sequence determination reactions were primed with the identical synthetic primers and templates that were used for the synthesis of the "run-off" transcripts described in the preparation and analysis of primer-extended products.

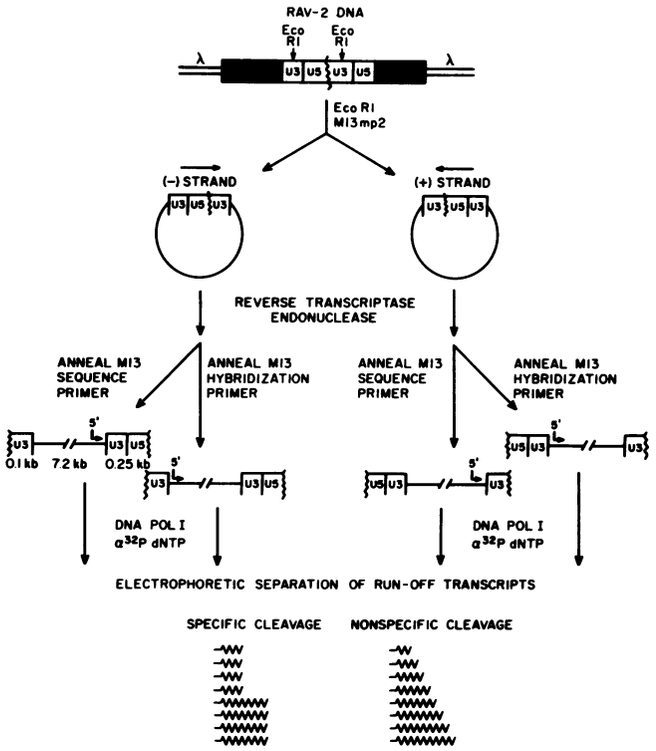

FIG. 1. Source of DNA substrates and fragments analyzed in a run-off assay of endonuclease-sensitive sites. The diagram at the top represents a recombinant clone containing an insert derived from unintegrated RF I retroviral DNA obtained from infected cells. The open area shows the tandem LTRs included in the insert with specific regions (U3 and U5) identified. The single-stranded LTR-containing substrates were derived from *Eco*RI subclones inserted in the two possible orientations in the M13 vector as indicated by the horizontal arrows (7). Each subclone contains a complementary strand (+ or −) of an entire LTR (in permuted order) plus the junction region between two LTRs indicated by a wavy line. These substrates were treated with the RT-endonuclease, and the products were analyzed by primer extension as described.

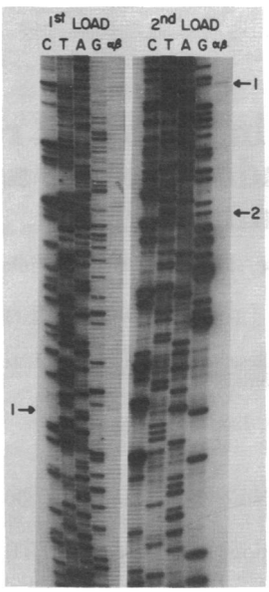

FIG. 2. Mapping of primary and secondary RT-endonuclease cleavage sites within the RAV-2 LTR (−) DNA strand. Circular single-stranded M13mp2 RAV2-2 (−) DNA was incubated for 30 min with the αβ form of ASV (Pr-C) RT-endonuclease in the presence of 5 mM Mn^{2+} in a first incubation. The sites of cleavage were then mapped by primer extension. The DNA sequence reads 5′ to 3′ from the bottom of the gel and directly corresponds to the sequence of the complementary (+) strand. Arrows indicate sites of cleavage for which sequence has been assigned unequivocally. Site 1 corresponds to the primary site of cleavage near the LTR junction. Site 2 is homologous to cellular DNA duplicated at the *ev*-1 provirus acceptor site (see the legend to Fig. 8A).

RESULTS

The Primer-Extension Assay. Fig. 1 illustrates the origin of the M13 LTR-containing clones utilized in these studies. Either the (+) or (−) strand of the viral DNA is represented, depending on the orientation of the insert. The primer-extension assay utilizes the oligonucleotide primers developed for M13 sequence and hybridization analyses. The sequence primer allows us to assay across the LTR insert; the hybridization primer

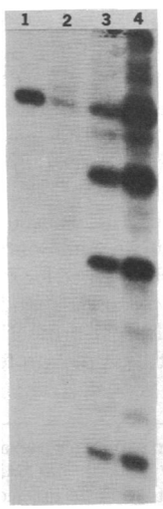

FIG. 3. Gel analysis of run-off fragments produced from M13mp2 RAV2-2 (−) DNA treated with RT-endonuclease or pancreatic DNase. Circular M13mp2 RAV2-2 (−) DNA was partially digested with the αβ form (lane 1) or ββ form (lane 2) of ASV (Pr-C) RT-endonuclease for 60 min, and primer extension analysis was performed as described. In a separate reaction, M13-mp2 RAV2-2 (−) DNA was incubated with 0.5 ng of pancreatic DNase (lanes 3 and 4) as described (5). In each case there was about 30% conversion from the circular to the linear form.

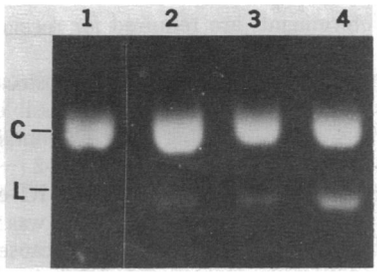

FIG. 4. Agarose gel electrophoresis analysis of M13mp2 RAV2-2 (−) DNA treated with the RT-endonuclease for increasing lengths of time. DNA was treated with the $\alpha\beta$ form of PrC-derived enzyme as described with incubation at 24°C, and the DNA was analyzed by gel electrophoresis (5). M13mp2 RAV2-2 (−) DNA was incubated 60 min without enzyme (lane 1) or with the RT-endonuclease for 15 min (lane 2), 30 min (lane 3), or 60 min (lane 4). C, position of single-stranded circular phage DNA molecules; L, position of single-stranded linear phage DNA molecules.

allows us to assay in a region of the vector adjacent to the LTR. Random cleavage generates a complex pattern of fragments; specific cleavages generate unique run-off fragments.

Initial studies revealed selective cleavages by RT-endonuclease as shown by the pattern in Fig. 2. There appeared to be several potential sites in the (−) DNA strand of the LTR when cleavage was carried out in Mn^{2+} concentrations > 1 mM. However, with the exception of one (no. 1 in Fig. 2), the pattern was not reproducible, and analysis of the (+)-strand run-offs did not identify the same sites. The exceptional fragment, identified in both (−)- and (+)-strand analyses, resulted from cleavage near the junction of LTRs. In experiments designed to optimize the reproducibility of the reaction, we found that the junction site was preferred for cleavage in the presence of a lower concentration of Mn^{2+}. The results described in the remainder of this report were obtained with these conditions.

Studies with the (−) DNA Strand of the LTR. M13mp2 RAV2-2 (−) DNA was digested with the $\alpha\beta$ or the $\beta\beta$ form of ASV RT-endonuclease. A single LTR run-off fragment was observed from DNA digested with the $\alpha\beta$ form (Fig. 3, lane 1). Two or three bands corresponding to a unique set of cleavage sites were obtained when DNA was treated with the $\beta\beta$ form (Fig. 3, lane 2; also data not shown); the major site appeared to be identical to that for the $\alpha\beta$ form, and a minor site appeared close by. For purposes of this study, the $\beta\beta$ enzyme was not considered further. Fig. 3 also shows the pattern obtained

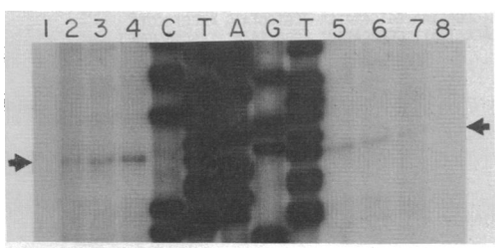

FIG. 5. Mapping a preferred RT-endonuclease cleavage site within the RAV-2 LTR (−) DNA strand. The remainder of samples prepared and monitored for cleavage as described in Fig. 4 were then mapped and analyzed by the primer-extension method. Dideoxynucleotide sequence analysis of M13mp2 RAV2-2 (−) DNA was carried out in parallel incubations and loaded onto the same gel. Labeling of transcripts and orientation of insert are as described in Fig. 2. The arrow indicates the unique primer-extension product. Lanes: 1 and 8, samples not treated with RT-endonuclease; 2–4, same samples shown in lanes 2–4 of Fig. 4; 7, 6, and 5, analogous to lanes 2, 3, and 4 except that B-77-derived enzyme was used. The site of cleavage, inferred from the sequence ladder, is shown in Fig. 8A.

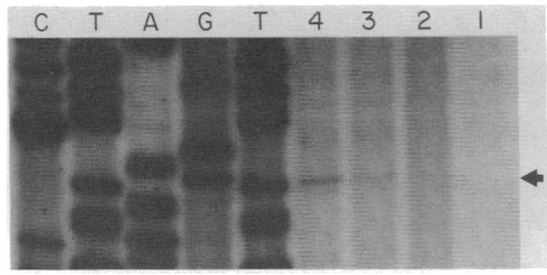

FIG. 6. Mapping the RT-endonuclease cleavage sites in the RAV-2 LTR (+) DNA strand. Circular M13mp2 RAV2-2 (+) DNA was incubated for increasing lengths of time with the $\alpha\beta$ form of ASV (Pr-C) RT-endonuclease, and primer-extension transcripts were prepared and analyzed as described in the legend to Fig. 2. M13mp2 RAV2-2 (+) DNA was incubated 60 min without enzyme (lane 1) or incubated with the RT-endonuclease for 15 min (lane 2), 30 min (lane 3), or 60 min (lane 4). The RT-endonuclease cleavage site inferred from the adjacent sequence assay ladder is indicated in Fig. 8A.

with DNA treated with pancreatic DNase. Many run-off fragments were observed, as expected for this relatively nonspecific endonuclease (Fig. 3, lanes 3 and 4). The varying intensities of these bands revealed some sequence preference. This is consistent with earlier studies on the specificity of this enzyme (10).

To demonstrate that the cleavages detected in the LTR were dependent on exposure to RT-endonuclease, M13mp2 RAV2-2 (−) DNA was incubated with either PrC or B-77 ASV endonuclease for increasing lengths of time. Aliquots were removed from the reaction, and the extent of cleavage of the DNA was determined by agarose gel electrophoresis. The results (Fig. 4) showed ≈5% conversion from circular to linear single strands in the shortest incubation time and ≈20% in the longest time. Therefore, under these conditions, each circular molecule cleaved had received, on the average, only a single break. We assume from previous studies that many of these breaks must be outside of the LTR and that the linear molecules represent a heterogeneous collection with permuted sequence (5). Those containing ends in the LTR were detected by subjecting the mixture to the primer extension analysis described above. The data (Fig. 5) show that the enzyme from both viruses cleaved primarily at one site within the LTR. The amount of the identifying fragment detected increased with time and verified its dependence on the RT-endonuclease. As a control (lanes 1 and 8), the M13mp2 RAV2-2 (−) DNA was incubated in the absence of the RT-endonuclease. No run-off fragment was detected. Thus, the fragment observed in this assay does not reflect natural pause sites for Pol I (Klenow fragment) or peculiarities of substrate secondary structure. The site for specific cleavage in the LTR as deduced from the sequence ladder is indicated in Fig. 8A.

Studies with the (+) Strand of the LTR. For these studies M13mp2 RAV2-2 (+) DNA was incubated with the $\alpha\beta$ form of RT-endonuclease until the extents of cleavage were similar to those shown in Fig. 4. Fig. 6 shows results of analysis with the PrC-derived enzyme; a similar pattern was obtained with the B-77 enzyme (not shown). The nucleotide sequence at the cleavage site is identical to that obtained with the (−)-strand template, and its relative position within a double-stranded LTR is indicated in Fig. 8A.

Site-Specific Cleavage Within the *lac* Region of the M13mp2 Viral Strand. Because RT-endonuclease does not require viral sequences in its substrate, it was of interest to determine the sequence of a cleavage site with the vector DNA. For these studies, the synthetic 15-base hybridization-probe primer was used. This primer hybridizes to the viral strand of M13mp2 and its derivatives at a region located 40 bases 5′ to the unique *Eco*RI

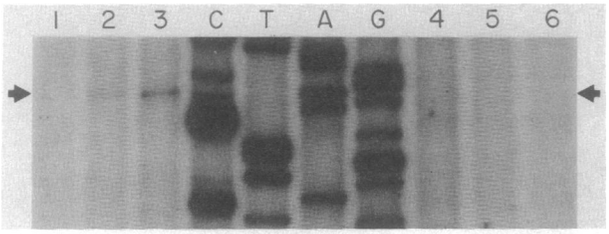

FIG. 7. Mapping an RT-endonuclease cleavage site within M13mp2 vector DNA. M13mp2 viral DNA or M13mp2 RAV2-2 (−) DNA was incubated with the $\alpha\beta$ form of ASV (Pr-C) RT-endonuclease for various lengths of time. Run-off products were prepared with the M13 hybridization primer and analyzed as described in the legend to Fig. 2. Run-off products were from M13mp2 DNA incubated with the RT-endonuclease for 0 min (lane 1), 30 min (lane 2), or 60 min (lane 3) and from M13mp2 RAV2-2 (−) DNA incubated with the RT-endonuclease for 60 min (lane 4), 30 min (lane 5), or 0 min (lane 6). The cleavage site inferred from the adjacent nucleotide sequence ladder is shown in Fig. 8*B*.

site used for inserting foreign DNA. Extension with Pol I allows analysis of a region adjacent to that into which an LTR would be inserted.

By using this assay, a time-dependent preferred cleavage site for the RT-endonuclease was identified within the 400-base region of the M13 vector DNA adjacent to the cloning site (Fig. 7, lanes 2 and 3). The site maps near the boundary of DNA from the *E. coli lac* region, which was built into the vector during its construction. The sequence at this site, which maps in the end of the *i* gene, bears no homology to the primary site identified in the LTR sequence (compare Fig. 8 *A* and *B*). However, it is also found within an inverted complementary repeat. As is the case for the site in the LTR, cleavage is a few bases 5′ to the axis of symmetry of the inverted repeat. We note that there are important transcription control regions (operator and promoter) and other inverted repeat sequences in the *lac* region scanned in this assay and that these are apparently not sites for cleavage. The latter observation suggests that additional, as yet undefined, determinants are required for recognition by the enzyme.

Because the hybridization primer binds upstream of foreign DNA inserts in the M13 vector, it was possible to monitor cleavages in this region by using the LTR-containing recombinants. Our analyses, using M13mp2 RAV2-2 (−) DNA, produced an unexpected result. The potential RT-endonuclease site revealed in the study of the vector DNA was not detected in the recombinant (Fig. 7, lanes 5 and 6). Because the enzyme was present in ≈40-fold molar excess to DNA, it seems unlikely that this result reflects limited enzyme concentrations. We note that the preferred *lac* DNA and the LTR cleavage sites in the recombinants are separated by ≈500 nucleotides, and this phenomenon could represent action at a distance (11, 12).

DISCUSSION

Through the use of a primer-extension assay and dideoxy sequence assay, we mapped specific sites in the RAV-2 retroviral LTR that are recognized by the endonuclease of the $\alpha\beta$ form of avian retrovirus reverse transcriptase. Earlier studies with RT-endonuclease revealed no preference for viral DNA; we are able to map LTR-specific cleavages only because this assay permits us to focus on selected regions of the substrate. These cleavage sites are noted in the sequence shown in Fig. 8*A*. In the presence of relatively high concentrations of Mn^{2+}, several sites were recognized in the (−)- and (+)-strand substrates. In many, but not all instances, they map in or adjacent to inverted complementary repeats. One site seems noteworthy because it occurs within a 6-base-pair sequence identical to that in the short host DNA duplication that apparently occurs as a consequence of integration of the endogenous avian retrovirus *ev*-1 (15). The relevance of this observation is difficult to assess, however, because this and all but one of the cleavage sites were not always detected. With lower concentrations of Mn^{2+}, one cleavage site in both the (−)- and (+)-strand substrate was preferred. Its location and the potential for generating a staggered cut in a dou-

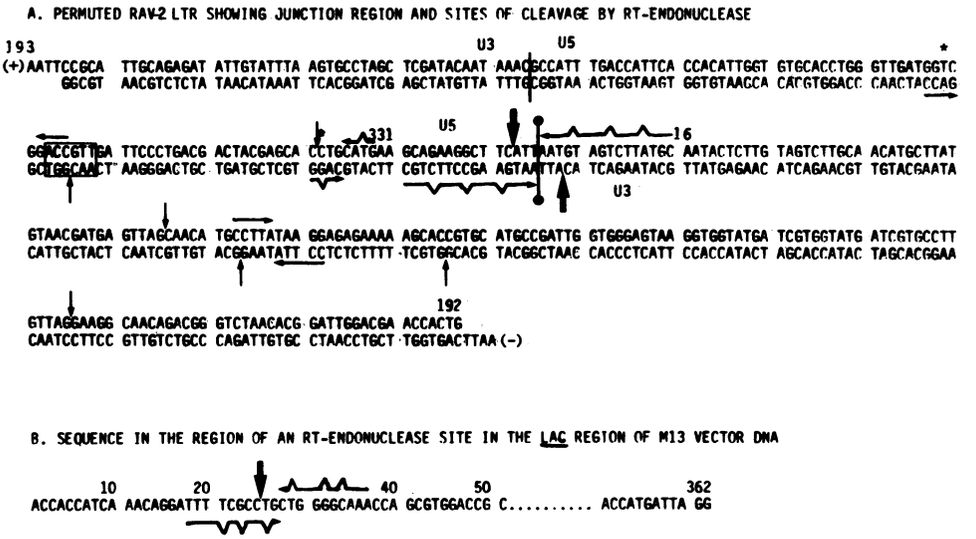

FIG. 8. Positions of RT-endonuclease cleavage sites in the RAV-2 LTR DNA sequence (*A*) and in the *lac* region of the M13mp2 viral DNA sequence (*B*). (*A*) The LTR is shown permuted around the *Eco*RI site (position 193) as it occurs in the M13 clones. Borders between the U3 and U5 regions are set off by vertical lines. The border at the LTR junction is marked by filled circles. Asterisks mark two nucleotides that differed when determining the sequence of clones that originally contained complementary strands. Although the substrates tested were single-stranded DNAs, complementary strands of the LTR sequence are shown to emphasize structural relationships. (*B*) The sequence numbering starts at the border of the section of *E. coli* DNA built into the vector during its construction (13). It ends 362 bases downstream at the edge of the *Eco*RI site used for cloning. Identification of these borders was made possible by comparing our data with information included in ref. 14. Primary cleavage sites are shown by the heavy vertical arrows; secondary cleavage sites are shown by light vertical arrows. The region of the LTR sequence homologous to the *ev*-1 cellular DNA acceptor site (15) is boxed. ←→, Inverted complementary repeats; ⌃, mismatch in the inverted complementary repeat.

ble-stranded DNA template is also shown in Fig. 8A. The cleavage is 5′ to the axis of symmetry in a 12/15-base-pair inverted complementary repeat that occurs at the junction of two tandem LTRs and that is presumed to be important in the integration reaction. This sequence configuration, found only in unintegrated RF I forms of viral DNA containing two tandem copies of the LTR sequence, is highly conserved among retroviruses (16–18).

Although the location of this preferred cleavage site suggests a role in integration, several features remain to be explained. The first relates to the actual points of cleavage. The termini of the LTRs of integrated provirus map 2 base pairs back from the LTR junction shown in Fig. 8A. Thus, if retroviral RF I DNA containing such a junction is a substrate for integration, the 4 intervening base pairs must be lost during that reaction. Under our conditions, RT-endonuclease cuts 3 base pairs back from the junction and, according to proposed models (19), would presumably result in a loss of 6 intervening base pairs. In more recent studies (unpublished data) using double-stranded substrates with nonpermuted LTRs, we found the same preferred cleavage site. Thus, our results do not seem attributable to use of an inappropriate substrate. We conclude, therefore, that if the observed sites of cleavage represent a correct intermediate along the integration pathway, additional steps must be required to generate the ends of the integrated provirus.

The nucleotide sequence of the cleavage site mapped in the *lac* region of the vector M13mp2 DNA is not homologous to the preferred site in the RAV-2 LTR. It is similar, however, in that it is found 5′ to the axis of symmetry in a nearly perfect (7/10) inverted complementary repeat (Fig. 8B). The lack of sequence homology in these two sites, the failure of the enzyme to cleave at similarly arranged inverted complementary repeats within the *lac* DNA analyzed, and the fact that the breaks that do occur may be displaced either two (*lac* DNA) or three (RAV-2 LTR) bases 5′ of the axis of symmetry again suggests that there exist additional undefined determinants in the reaction. This pattern of activity in which cleavage is not simply dictated by a unique nucleotide sequence but occurs preferentially at a subset of cleavage sites is reminiscent of that observed for DNA gyrase-catalyzed cleavage of double-stranded DNA in the presence of oxolinic acid (20). We note also that spacings inferred for the overlap in sites of cleavage during the transposition of prokaryotic transposable elements can also be variable (21, 22).

Aside from its possible relevance to integration, the mapping of cleavage sites allows comparison with other enzymes of similar activity. The capacity to cleave single-stranded DNA site specifically is also demonstrated by several restriction endonucleases including *Hae* III, *Hha* I, *Rsa* I, and *Taq* I (23, 24). As with these restriction enzymes, the RT-endonuclease appears to cleave at the same sites recognized in double-stranded (RF I) substrates (unpublished data). Cleavages introduced by the RT-endonuclease at increased divalent cation concentrations may be analogous to the illegitimate sites observed using the aforementioned restriction endonucleases on single-stranded DNA substrates (25).

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