JOURNAL OF VIROLOGY, Nov. 1985, p. 589–599
0022-538X/85/110589-11$02.00/0

Vol. 56, No. 2

Circles with Two Tandem Long Terminal Repeats Are Specifically Cleaved by *pol* Gene-Associated Endonuclease from Avian Sarcoma and Leukosis Viruses: Nucleotide Sequences Required for Site-Specific Cleavage

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Received 7 May 1985/Accepted 6 July 1985

The avian retroviral *pol* gene-encoded DNA endonuclease (*pol*-endo) has been shown to selectively cleave the viral long terminal repeat sequences (LTRs) in single-stranded DNA substrates in a region known to be joined to host DNA during integration (G. Duyk, J. Leis, M. Longiaru, and A. M. Skalka, Proc. Natl. Acad. Sci. USA 80:6745–6749, 1983). The preferred sites of cleavage were mapped to the unique U5/U3 junctions found only in covalently closed circular DNA molecules containing two tandem LTRs. The cuts occurred three nucleotides 5′ to the axis of symmetry of the 12-of-15-base-pair nearly perfect inverted repeat which marks the LTR junction. Experiments with double-stranded supercoiled DNA substrates revealed a similar specificity for nicking. Also, the endonuclease associated with the *pol* cleavage product, pp32, has the same specificity as the αβ form. The limits of sequence required for site-selective cleavage near the U5/U3 junction were established with single-stranded DNA substrates. A domain no larger than 44 base pairs allowed site-selective cleavage in each strand in vitro. Recognition of either strand appeared to be independent of the other, and in each case, the critical sequence was asymetrically distributed with respect to the U5/U3 junction. The predominant contribution was from the U5 domain; this is consistent with its conservation in the LTR sequences of a number of avian sarcoma and leukosis viruses.

Integration of retroviral DNA into the host-cell genome is a normal step in the replication of retroviruses. Analyses of the structure of integrated and unintegrated viral DNA reveal the following. (i) The integration reaction is specific for sites near the ends of the viral long terminal repeats (LTRs) but relatively nonspecific for sequences in the host DNA. (ii) There is a short duplication of host sequences at the site of integration which is characteristic of a particular virus and not of the host cell in which it grows (28). (iii) Naked retroviral DNA introduced into host cells by transfection or microinjection is not integrated properly (13). Our recent studies of the DNA endonuclease associated with the avian retroviral reverse transcriptase (*pol*-endo) suggest that the viral *pol* gene encodes an activity required for proviral DNA integration (4).

The predominant form of reverse transcriptase isolated from avian sarcoma and leukosis virus (ASLV) particles contains two polypeptide chains, α and β, with molecular masses of 61,000 to 63,000 daltons and 91,000 to 93,000 daltons, respectively. The β chain is encoded in the *pol* gene. The α chain is produced from β by a proteolytic cleavage which releases a 32,000-dalton phosphoprotein, pp32 (5, 8, 20, 21). Endonuclease activity is associated with the αβ and ββ forms of the enzyme and with pp32, but not with the α chain (6, 7, 12). This indicates that the active site for the endonuclease activity is in the carboxyl domain of the β chain as defined by pp32. The αβ form requires Mn^{2+} for activity in vitro, whereas the ββ form or pp32 utilizes either Mg^{2+} or Mn^{2+}. The preferred DNA substrates are replicative form I (RF I) and single-stranded DNA (12). Many single- and some double-strand breaks with 3′ OH and 5′ P termini are introduced into DNA.

By use of a primer extension assay and single-stranded DNA substrates, the αβ form of the ASLV *pol*-endo was shown to selectively cleave viral LTRs near the region joined to host DNA during integration (4, 24). The sites of cleavage map in a unique 12-of-15-base-pair nearly perfect inverted repeat (IR) sequence formed by the juxtaposition of the termini of two tandem LTRs. This specificity strongly suggests that the ASLV *pol*-endo plays a role in integration. It predicts that *pol* gene mutations in the endonuclease domain block integration, that the IR sequences at the termini of the unintegrated viral DNA are essential for integration, and that the circular unintegrated form of viral DNA containing two tandem copies of the LTR is a precursor to integration. All of these predictions have recently been verified experimentally (3, 17–19, 22).

In this report, we show that the ASLV *pol*-endo has the same cleavage site specificity in single-stranded and RF I DNA substrates and that under our conditions the specificity of the αβ and pp32 forms of the endonuclease are indistinguishable. The limits of sequence required for site-specific cleavage at the tandem junction of two LTRs in single-stranded substrates were established. Our analyses indicate that the *pol*-endo cleaves a palindromic DNA sequence symmetrically but recognizes it asymetrically.

MATERIALS AND METHODS

Reagents. [α-^{32}P]dCTP (600 to 800 Ci/mmol) was purchased from New England Nuclear Corp., Boston, Mass. [γ-^{32}P]ATP (7,000 Ci/mmol) was purchased from ICN Radiochemicals, Irvine, Calif. DNA polymerase I (Klenow fragment), T4 DNA ligase, T4 polynucleotide kinase, Isopropyl-β-D-thiogalacto-

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pyranoside, 5-bromo-4-chloro-3-indolyl-β-*O*-galactoside (X-gal), and restriction enzymes were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Pancreatic DNase I and hen egg white lysozyme were purchased from Worthington Life Sciences Division, Freehold, N.J. BAL 31 nuclease (slow) was purchased from International Biotechnologies, Inc. M13 17-mer sequencing primer, pBR322 15-mer *Eco*RI site primer, and pBR322 16-mer *Hin*dIII site primer were purchased from New England BioLabs, Inc., Beverly, Mass. Acrylamide, bisacrylamide, and N,N,N',N'-tetramethylethylenediamine were purchased from Bio-Rad Laboratories, Richmond, Calif. Urea (analytical reagent) was purchased from BDH Chemicals Ltd., Poole, England. Agarose was from FMC Corp., Marine Colloids Div., Rockland, Maine. Ethidum bromide was from Calbiochem-Behring, La Jolla, Calif. Deoxynucleotides and dideoxynucleotides were purchased from Pharmacia, Uppsala, Sweden, and P-L Biochemicals, Inc., Milwaukee, Wis. Preparative-grade cesium chloride was purchased from Var Lac Oid Chemical Co. Nitrocellulose hybridization membranes (HAHY) were from Millipore Corp., Bedford, Mass. Preparative C-18 resin was from Waters Associates, Inc., Milford, Mass.

DNA preparations. Permuted LTR subclones prepared from segments of Rous-associated virus-2 (RAV-2) retroviral DNA have been described previously (10, 11). RF I supercoiled DNA free of contamination with other forms was prepared by purification through two cesium density gradients and subsequent extraction with acidified phenol by the method of Zasloff et al. (30). Covalently closed circular single-stranded DNA, free of linear DNA, was prepared by velocity sedimentation through high-salt sucrose density gradients as described by Griffith and Kornberg (9). M13 phage and RF I DNA were prepared as described by Zinder and Boeke (31). M13mp2 RAV-2-2 and natural deletion clones RAV-2-12, Schmidt-Ruppin strain B (SRB) *td*7, and SRB *td*9 were previously described (10).

Purification of reverse transcriptase. Purification of the αβ form of avian myeloblastosis virus (AMV) reverse transcriptase was as previously described (12). AMV pp32 was a generous gift of D. Grandgenett, St. Louis University, St. Louis, Mo.

Endonuclease assay conditions. DNA endonuclease activity was monitored as described by Leis et al. (12). Digestion of 5′ ^{32}P-labeled oligonucleotides was done under the conditions outlined by Duyk et al. (4). At least 0.1 pmol of oligonucleotide was digested in each reaction. In digests of cloned single-stranded DNA, 0.5 to 1.0 μg of M13 DNA containing retrovirus LTR inserts was incubated with various amounts of αβ *pol*-endo as indicated in a volume of 10 μl. For pp32, 5 to 15 ng of protein was incubated with 1.45 μg of DNA in the same volume. The mixture was incubated at 24°C for 2 min, and then 2 μl of either 6 mM $MgCl_2$ or 6 to 12 mM $MnCl_2$ was added. Incubation was continued at 37°C for various lengths of time, and the reaction was stopped by heating at 65 to 70°C for 3 min.

The endonuclease assay conditions for RF I DNA were as follows. AMV αβ *pol*-endo (2 polymerase units) was incubated in a final volume of 10 μl which contained 20 mM Tris hydrochloride (pH 8.4), 5 mM 2-mercaptoethanol, and 500 ng of double-stranded supercoiled plasmid DNA. The mixture was incubated for 2 min at room temperature, and then 2 μl of 6 mM $MnCl_2$ was added to the reaction to bring the final volume to 12 μl. After incubation at 37°C for lengths of time as indicated, the reaction was stopped by heating to 65°C for 5 min and then placed on ice. A 2-μl sample was removed to determine the extent of nicking by visualizing the RF I-to-RF II DNA conversion on agarose gels. The remainder of the DNA was used for the primer extension reaction.

Preparation and analysis of primer extension reactions. The primer extension assay for single-stranded DNA was as previously described (4). Double-stranded DNA from *pol*-endo digestions was ethanol precipitated after the addition of EDTA to 10 mM. The pellets were suspended in 8 μl of water, and *Bam*HI and buffer were added to a final volume of 10 μl. *Bam*HI cleavage was used to linearize the DNA before annealing to oligodeoxynucleotide primers. After digestion for 60 min at 37°C, 1 μl was loaded onto an agarose gel to verify that all the substrate DNA was linearized, and the remainder of the reaction was used for the primer extension analysis. A 1-μl portion of 10× HIN buffer (66 mM Tris hydrochloride [pH 7.5], 66 mM $MgCl_2$, 500 mM NaCl, 50 mM dithiothreitol) and 5 ng of single-strand primer were added to the reaction. This mixture was heated at 90°C for 5 min and then plunged directly into an ice-water bath. To monitor cleavage sites on each DNA strand, either the pBR322 *Hin*dIII site primer (GCAATTTAACTGTGAT) or the *Eco*RI site primer (GTATCACGAGGCCCT) was annealed to the DNA from a single *pol*-endo digestion reaction. Primer extension was carried out by the addition of 4 μCi of [α-^{32}P]dATP, 1 U of DNA polymerase I (Klenow fragment), the other three deoxynucleotides, each to a final concentration of 10 μM, and dithiothreitol to a final concentration of 6 mM. The mixtures were incubated for 15 min at 37°C. The reactions were stopped and then analyzed by polyacrylamide gel electrophoresis as described by Maxam and Gilbert (15). Dideoxynucleotide sequencing ladders were run in parallel, using the same DNA and primers, to map the cleavages introduced by the endonuclease. Figure 1 provides a summary of the assay procedures.

Preparation of synthetic oligonucleotides. Oligonucleotides were synthesized on a solid support by the phosphoramidite method (1, 2). Oligonucleotides were purified from crude synthesis mixtures by electrophoresis on 20% polyacrylamide gels. After gel elution, the DNAs were separated from residual gel material on small C-18 columns.

Cloning of oligodeoxynucleotides. The synthetic junction fragments 20-mer and 30-mer wild type (wt) (see Fig. 3) were cloned in the *Sma*I site of M13mp19. Ligations were performed overnight at 15°C at a 20:1 molar excess of insert to *Sma*I-digested vector DNA (40 ng), using 1 U of T4 DNA ligase in the presence of 20 mM Tris hydrochloride (pH 7.5)–10 mM $MgCl_2$–10 mM dithioerythritol–0.6 mM ATP. Putative recombinants were selected by failure of α-complementation of β-galactosidase and identified by dot-blot hybridization with 5′ ^{32}P-labeled 20-mer plus or minus strands as probes as described by Messing (16) with the following modifications. The filters were prehybridized for 2 to 3 h at 55°C in 20 ml of 0.9 M NaCl–90 mM sodium citrate–0.2% sodium dodecyl sulfate–0.1% Ficoll–0.1% polyvinylpyrrolidone–10 mg of bovine serum albumin per ml–100 μg of sonicated roe DNA per ml. The filters were then hybridized overnight at room temperature in 10 ml of 0.9 M NaCl–90 mM sodium citrate–0.2% sodium dodecyl sulfate–20 μg of tRNA per ml and labeled probe. Filters were washed six times with 0.9 M NaCl–90 mM sodium citrate–0.2% sodium dodecyl sulfate before putative recombinants were identified via autoradiography. The presence of appropriate inserts was confirmed by dideoxynucleotide sequencing. The 20-mer double-stranded insert was prepared by mixing equimolar amounts of the complementary

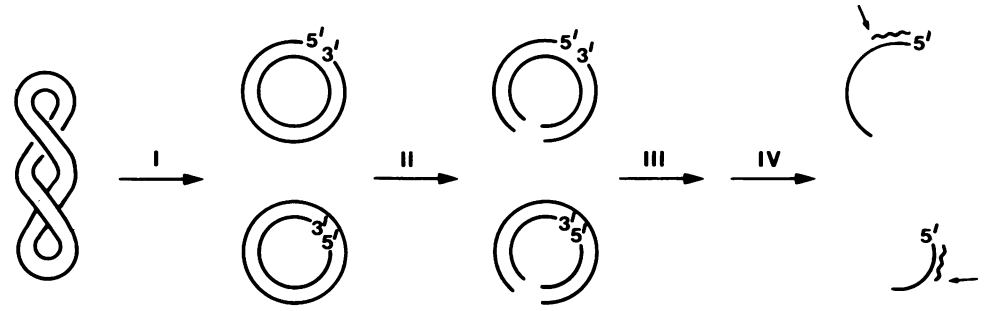

FIG. 1. Primer extension assay for mapping breaks in RF I DNA substrates. (I) RF I DNA is incubated with the *pol*-endo for various lengths of time producing nicked open circles (RF II). (II) RF II products are digested with a restriction endonuclease which cuts only once within the vector DNA to produce linear duplex DNA (RF III). (III, IV) The linearized DNA is then denatured and annealed to single-strand oligodeoxynucleotide primers at sites designated by the arrows. Components for the primer extension reaction are then added to primed DNA, and runoff fragments are synthesized and analyzed as described in Materials and Methods.

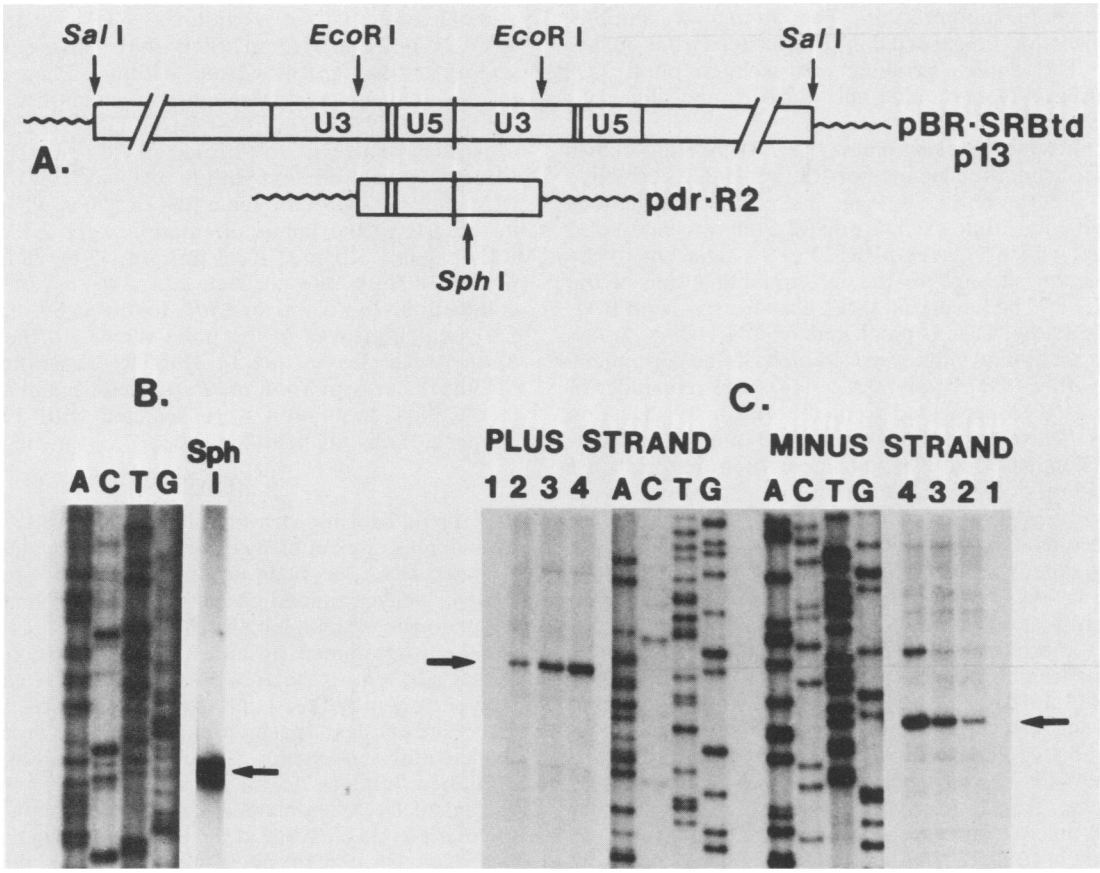

FIG. 2. Gel analysis of primer-extended products from *Sph*I-cleaved and *pol*-endo-cleaved DNA containing a permuted RAV-2 LTR. (A) The diagram provides an abbreviated map of two plasmids utilized in our studies and shows the relationship of their LTR sequences to each other. The pBR-SRB*td*-p13 plasmid is a molecular clone that includes a complete viral genome permuted around a unique viral *Sal*I site inserted into the unique *Sal*I site of pBR322. The pdr-R2 plasmid contains an *Eco*RI subsection from the tandem LTRs inserted into the unique *Eco*RI site of pBR322. The wavy line indicates pBR322 sequences adjacent to the inserts. (B) The RF I of pdr-R2 DNA was linearized with *Sph*I, denatured, and annealed with the unique *Hin*dIII-site single-strand pBR322 primer as described in Materials and Methods. Primer-extended products were loaded onto sequencing gels and run in parallel with dideoxynucleotide sequencing ladders formed with the same template and primer. (C) The RF I form of pdr-R2 was incubated with the AMV αβ *pol*-endo, and primer extension products were analyzed as in panel B. Lane 1 is a control reaction with pdr-R2 RF I DNA and no endonuclease. Lanes 2 to 4 show results after incubation for increasing lengths of time with AMV αβ *pol*-endo. Incubations were carried out at 32°C for 2 min (lane 2), 5 min (lane 3), and 15 min (lane 4). The plus-strand primer extensions and sequencing were made with the unique *Eco*RI-site single-strand primer for pBR322, while the minus-strand extensions were from the *Hin*dIII-site single-strand primer. The primer-extended fragments which reveal breaks near the U5/U3 junction are marked by the arrows. C, T, A, and G indicate the dideoxynucleotide sequencing ladders. See Materials and Methods for details. The DNA sequence and primer-extended products read in a 5′-to-3′ direction from the bottom to the top of the gel and directly correspond to the sequence of the complementary strand.

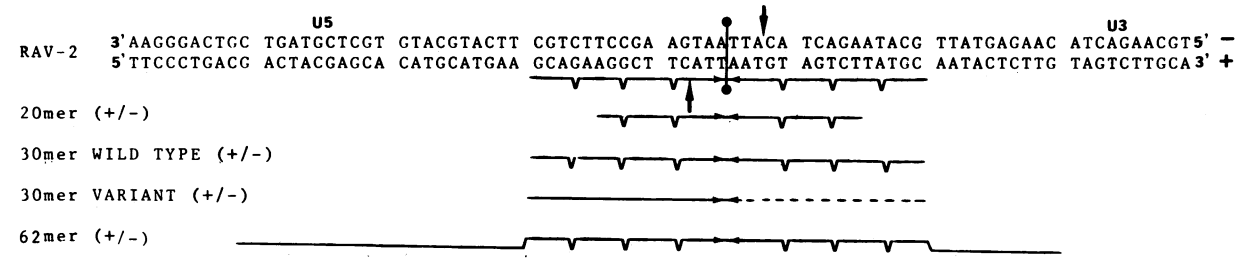

FIG. 3. Synthetic oligodeoxynucleotides corresponding to the RAV-2 LTR U5/U3 junction region. A portion of the nucleotide sequence of the RAV-2 LTR about the U5/U3 junction is presented with vertical arrows showing the *pol*-endo-specific cleavage sites. The vertical line capped by closed circles (●) defines the junction. The horizontal lines indicate the oligonucleotides synthesized; (+/−) indicates that both complementary strands were made. The horizontal arrows indicate that all or part of the 12-of-15-nucleotide IR sequence is included in the oligonucleotides, and the breaks in the lines show noncomplementary nucleotides. The broken horizontal arrows in the case of the 30-mer var indicate that this substrate is composed of perfect inverted complementary U5 repeats.

fragments in 50 mM Tris hydrochloride (pH 7.5)–10 mM $MgCl_2$, heating the mixture to 90°C for 3 min, and slow cooling to room temperature. The 30-mer wt double-stranded insert was constructed in several steps from smaller oligomers. The duplex product was isolated on a 12% nondenaturing polyacrylamide gel, purified, and cloned as described above.

BAL 31 nuclease deletion clones. BAL 31 deletion clones were derived from the plasmid pdr-R2, a pBR322 derivative containing a permuted LTR (Fig. 2A). The constructions were carried out as follows. The pdr-R2 clone was linearized with *Hin*dIII which cleaves pBR322 DNA adjacent to the *Eco*RI insertion site and to the U5-containing side of the permuted LTR. The linearized DNA was digested with BAL 31 nuclease (slow) (1.6 U/pmol end of DNA) for various times (29), and blunt ends were created at the termini by treatment with T4 DNA polymerase (14). The remainder of the LTR was then separated from the vector by digestion with *Eco*RI. These fragments were cloned in *Eco*RI-*Hin*dIII-digested M13mp18 DNA. (The final constructs are displayed in Fig. 5.) Clones containing junction sequence inserts were selected by hybridization with the 5′ ^{32}P-labeled 20-mer plus-strand oligodeoxynucleotide (Fig. 3). Deletions were mapped by dideoxynucleotide sequencing of the recombinant phage DNAs. Under these conditions, the distribution in size of products was broad, indicating an average rate of digestion of approximately 7 to 10 bases per min. Deletions of the U3 portion of the LTR were constructed from the RF I form of M13mp18:4(−) (see Fig. 5). DNA was linearized at the *Eco*RI site just 5′ to the insert and then digested with BAL 31 as above. The DNA was digested with *Cla*I and was then treated with T4 DNA polymerase followed by *Hin*dIII (which cleaves only in the vector) to separate the remainder of the LTR insert from the mp18 vector. The mixture was then ligated to *Hin*dIII-*Hin*dII-digested M13mp19, and the recombinant molecules were introduced into the M13 bacterial host, JM101. The desired recombinants (clones 11, 12, 8, and 9 [see Fig. 7]) were identified by using 5′ ^{32}P-labeled U3-oligodeoxynucleotide probes (X [GCACGGTGCTTTT-TCTCTCC], Y [TCATCGTTACATAAGCATGT], and Z [GCATAAGACTACATTAATG]).

Preparation of cloned viral inserts: changing the orientation of selected clones. Our strategy for reversing the orientation of selected clones took advantage of restriction enzyme sites adjacent to the inserts which are part of the polylinkers of the cloning vectors. All minus- and plus-strand clones are listed in Fig. 5 and 7, respectively. Several of them were digested to completion with *Cla*I or *Xmn*I (which cuts only in the vector), and blunt ends were created with T4 DNA polymerase. The inserts were then released from the parent molecules and cloned into the appropriate restriction enzyme-digested vector as follows. *Cla*I-digested M13mp2-RAV-2-12(−) and M13mp2:SRB *td*9(−) were cut with *Eco*RI and ligated to *Eco*RI-digested M13mp7. Phage maintaining inserts in the plus orientation were identified with the 5′ ^{32}P-labeled U5-(CGTGTTAGACCCGTCTGTTG) oligonucleotide probe. *Cla*I-digested M13mp19:8(+) and M13mp19:9(+) were cut with *Eco*RI and *Hin*dIII and ligated to M13mp8 cut with the same two enzymes. Phage maintaining inserts in the minus orientation were selected with 5′ ^{32}P-labeled 20-mer (+) probe. The RF DNA of M13mp18:5(−), :6(−), :7(−), and :14(−) were digested to completion with *Xmn*I or *Cla*I, followed by digestion with *Pst*I (which cleaves in the polylinker 3′ to the insert) and *Sph*I (which cleaves in U3). This DNA was then ligated to M13mp18 cut with *Sph*I and *Pst*I. Phage maintaining inserts in the plus orientation were selected with 5′ ^{32}P-labeled 20-mer (−) oligonucleotide probe.

RESULTS

Mapping *pol*-endo cleavage sites in RFI DNA. The method for mapping sites of cleavage in particular regions of single-stranded DNA has been described in detail previously (4). Briefly, single-stranded circular DNA is incubated with the *pol*-endo for various lengths of time. The sites of cleavage are then determined by annealing a short complementary primer adjacent to the area of interest and extending it with DNA polymerase I (Klenow) and α-^{32}P-labeled deoxynucleotides. Specific runoff fragments are analyzed on a nucleotide-sequencing gel adjacent to dideoxynucleotide sequence ladders formed with the same primer and uncleaved DNA template. Using this protocol, a preferred site of *pol*-endo cleavage at the junction of two tandem LTRs was demonstrated in single-stranded DNA substrates (4). Supercoiled DNA is a preferred substrate for the various forms of the *pol*-endo (6, 12). Since RF I provirus DNA has been identified as a replication intermediate in vivo (28), it was important to determine if the *pol*-endo would cleave near the tandem LTR junction in this DNA form. Mapping of cleavage sites in RF I DNA substrates can be carried out by a modification of the primer extension method (24) (Fig. 1). The RF I DNA is cleaved to RF II DNA by the *pol*-endo. The DNA is then linearized with a restriction enzyme, denatured, and annealed to an oligodeoxynucleotide primer. By using primers complementary to each strand, plus- and minus-strand cleavage sites can be mapped on the same DNA molecule.

***pol*-endo nicks LTRs at the same sites in RF I and single-**

stranded DNA substrates. The RF I DNA used for these studies is pdr-R2, which contains an *Eco*RI-permuted RAV-2 LTR (Fig. 2A). It is similar to the M13mp2 derivatives described in previous *pol*-endo studies (12) except that the LTR fragment is inserted into the unique *Eco*RI site of pBR322. Figure 2B shows the results of a control experiment in which pdr-R2 was digested with *Sph*I, which cuts at a unique site within U3. Under these conditions, a single runoff fragment was observed. Its position correctly identified this restriction endonuclease site, thus verifying the accuracy of the mapping procedure. The intensity of the runoff band also served as an internal control indicating the signal expected if a particular site was cut at 100% efficiency.

The RF I pdr-R2 DNA was then treated with the αβ form of AMV *pol*-endo for increasing amounts of time, and the extent of cleavage was monitored by agarose gel electrophoresis (data not shown). The conversion of RF I to RF II DNA varied from 30% at the shortest to 90% at the longest time of incubation. The results of the mapping studies show a prominent runoff fragment whose amount increased with the extent of substrate nicking (Fig. 2C). The intensity of the bands is severalfold less than that observed for the *Sph*I-cut DNA. The cleavage sites, determined from the adjacent sequence ladders, occur in the same sequence on each strand and are identical in location to those observed with single-stranded substrates (Fig. 2C). The data for the minus-strand analysis in Fig. 2C show additional runoff fragments of lower intensity which map downstream from the major site. These secondary sites have been observed in single-stranded substrates, and their significance is unknown.

The above experiments were carried out with the *Eco*RI-permuted LTR DNA. Since it was possible that this arrangement of DNA might influence the choice of the *pol*-endo cleavage site, the experiment was repeated with RF I DNA containing two complete tandem LTRs in a nonpermuted orientation (pBR-SRB*td*-p13, Fig. 2A). To map cleavage sites in this substrate, primers complementary to the viral DNA sequence adjacent to the LTR were synthesized, one for each strand. The αβ form of AMV *pol*-endo was found to nick this substrate at the same locations as molecules with the permuted sequence (data not shown). Thus, all the information required for recognition and cleavage appears to be contained within the junction region irrespective of the overall arrangement of the LTR DNA. Furthermore, since RF I and single-stranded DNA substrates gave the same results, we conclude that the specificity of this endonuclease may be assayed with either substrate.

The IR segments alone are not sufficient to support site-specific cleavage in vitro. The junction region in molecules with tandem LTRs is unique because it juxtaposes sequences which are not adjacent in linear integrated or linear unintegrated DNA. As such, it contains a new arrangement which is an attractive candidate for recognition by enzymes of an integration system. Common to all unintegrated retroviral circular DNAs with intact junction regions is a palindromic sequence composed of slightly imperfect IRs of various lengths (see Fig. 10B). At the core of this region lies the highly conserved sequence 5′-CATTAATG-3′, in which the underlined bases are destined to form the ends of the integrated provirus. The simplest hypothesis to account for the identity of the cleavage sites at the U5/U3 junction is that the enzyme utilizes the 30-base palindromic sequence or some subset of it as both a recognition and cleavage site.

To test this hypothesis, a series of oligodeoxynucleotides corresponding to the U5/U3 junction region of tandemly arranged LTRs of RAV-2 were synthesized as described in Materials and Methods (Fig. 3). These include oligonucleotides corresponding to either strand of the entire wt 30-base palindromic sequence (30-mer wt) (+/−) and a 20-base subset of it (20-mer wt) (+/−) also centered about the U5/U3 junction. In addition, a variant (var) 30-base junction fragment (30-mer var) (+) representing a perfect IR formed from the U5 IR plus-strand sequence was prepared. To test for specific cleavage, each fragment was labeled with ^{32}P at its 5′ end and incubated with the αβ form of AMV *pol*-endo. The products of the reaction were then separated by polyacrylamide gel electrophoresis. The results show (Fig. 4) that both the 20-mer and 30-mer wt fragments were cleaved poorly, if at all. In contrast, the appearance of a small amount of a ^{32}P-labeled 12-nucleotide fragment in the reaction with the 30-mer var (+) indicated that it was cleaved to some extent at the same site observed, using a more complete copy of the LTR and the primer extension assay. The significance of this result, which suggests that alteration in the symmetry of the palindromic sequence influences its cleavage by the *pol*-endo, is not known. Some site-specific cleavage (approximately 15%) was also obtained with a 62-nucleotide oligomer which spans the junction region (Fig. 4). Cleavage at other sites was detected, but to a much lower extent than at the specific site.

To test the hypothesis that the failure of the endonuclease to cleave the 30-mer wt oligomers was a consequence of the limited size of these fragments, a duplex of the fragment was cloned in either direction in the *Sma*I site of M13mp19 RF. The resultant recombinant single-stranded phage DNAs were assayed for cleavage by primer extension as described above. Specific cleavage near the U5/U3 junction was not observed regardless of orientation of the insert (data not shown). The 30-mer var could not be tested because stable clones were not obtained. The 30-mer wt duplex fragment was also inserted into a vector downstream from the RAV-2 *Eco*RI-permuted LTR sequence containing specific cleavage sites. When this arrangement was assayed by the primer extension method, cleavage was observed within the permuted LTR but not within the 30-mer wt segment (data not shown). Thus, proximity to a recognized cleavage site did not facilitate cleavage of the 30-mer wt fragment.

Determination of the limits of sequences required for recognition and specific cleavage by *pol*-endo near the U5/U3 RAV-2 LTR junction. The results of tests with synthetic oligonucleotides suggested that information in addition to the IRs is required for site-specific cleavage. To locate the borders of the required region, a series of BAL 31 nuclease deletions were constructed with the *Eco*RI-permuted RAV-2 LTR contained in the RF I forms of various M13 recombinants as outlined in Materials and Methods. The initial mutants contained deletions of the U5 sequence beginning at locations distal to the U5/U3 junction and proceeding toward it (Fig. 5). Single-stranded circular phage DNA from each deletion mutant was analyzed for specific cleavage by the *pol*-endo by the primer extension assay. When the limit of sequence required for specific cleavage was established, the RF I DNA of the corresponding clone was used to create deletions on the U3 side of the junction (Fig. 8). In some cases, it was necessary to change the orientation of the deleted inserts through a second cloning step. In addition to the BAL 31 deletions, a series of naturally occurring deletion mutants of the U3 region isolated by Ju et al. (10) were subcloned into M13 vectors and used for primer extension assays.

Analysis of requirements for minus-strand cleavage. Results from the set of experiments which established the

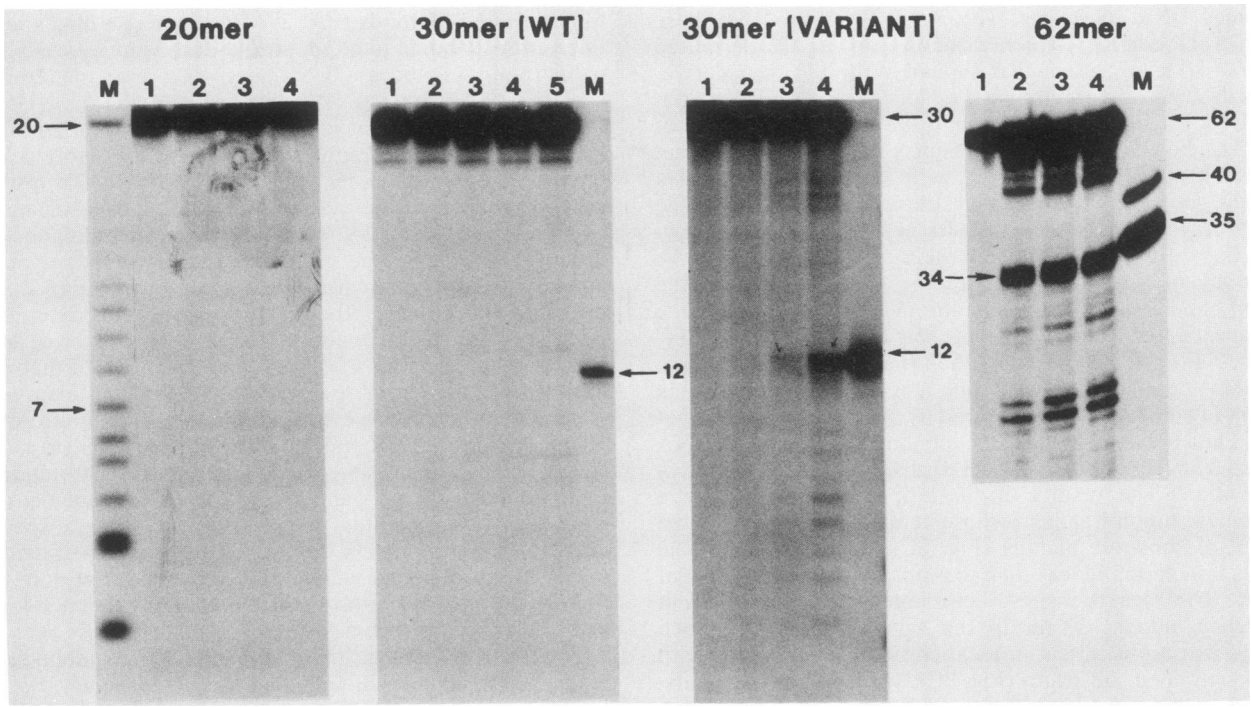

FIG. 4. Test of the sensitivity of junction oligodeoxynucleotides to *pol*-endo cleavage. AMV αβ *pol*-endo was incubated with 0.3 pmol of the indicated oligodeoxynucleotides which had been enzymatically labeled with ^{32}P at their 5′ termini with T4 polynucleotide kinase as described in Materials and Methods. The products were analyzed by electrophoresis in a 20% denaturing polyacrylamide sequencing gel. Lane M in the 20-mer analysis contains a snake venom diesterase ladder of the labeled oligomer. Arrows indicate the position of 20-mers (unreacted material) and 7-mers (the predicted 5′ ^{32}P-labeled product produced by the site-specific cleavage of the 20-mer). Lane M in the 30-mer (wt and var) analysis contains a 12-mer marker which designates the expected site-specific cleavage fragment of the 30-mer. In the 20-mer and 30-mer (var) experiments, lane 1 shows the products of an incubation in the absence of enzyme; lanes 2, 3, and 4 show products of an incubation at 37°C in the presence of enzyme for 30, 60, and 90 min, respectively. In the 30-mer wt (+) analysis, lane 1 shows the product of an incubation in the absence of enzyme; lanes 2, 3, 4, and 5 show reactions incubated with the *pol*-endo for 15, 30, 60, and 120 min, respectively. In the 62-mer analysis, lane M shows markers of 40 and 35 nucleotides in length. Lanes 2, 3, and 4 show products from *pol*-endo reactions incubated for 20, 40, and 60 min, respectively.

sequence limits for cleavage in the minus strand are summarized in Fig. 5. The difference observed between clones 4 and 5 indicates that between 29 and 36 nucleotides on the U5 side of the junction are required for cleavage. Data for this comparison are included in Fig. 6. It should be noted that a new cryptic site occupying the same relative position as the *pol*-endo cleavage site was not observed in clone 5.

Comparison of results with clones 8 and 9 indicates that only three to eight nucleotides are required on the U3 side of the junction. The negative result with clone 9, which contains two bases on the U3 side of the junction, was confirmed by results from analysis of the naturally occurring deletion mutants SRB *td*-7, SRB *td*-9, and RAV-2-12. Thus, we conclude that the upper limit of sequence required for *pol*-endo recognition and cleavage within the minus strand is 44 nucleotides. The larger portion of this sequence is derived from U5 and includes at least a portion of the palindrome formed by this junction.

Analysis of requirements for plus-strand cleavage. The DNA insert containing the limit sequence required for specific cleavage of the minus strand, M13mp8:8(−), was recloned in reverse orientation to obtain single-stranded DNA containing the plus-strand junction sequence. This DNA, M13mp19:8(+), was a substrate for cleavage near the U5/U3 junction (Fig. 7). Surprisingly, further analyses (summarized in Fig. 7) indicated that the length and nature of the sequence required for cleavage of the plus strand were not identical to those observed for the minus strand. This is illustrated by the fact that M13mp19:9(+) (U5, 36/U3, 2) was cleaved at the expected site, whereas its complement M13mp8:9(−) (U3, 2/U5, 36) was not (see Fig. 5 and 7). This result was confirmed by using the natural mutants whose deletions start exactly at the U3/U5 boundary (SRB *td*7, RAV-2-12, and SRB *td*9 [11]); all three of these mutants were cleaved specifically. The data for the SRB *td*(+) analysis are shown in Fig. 8A. Analysis of the recombinant clone 13(+) (Fig. 7), whose deletion includes the junction and the specific cleavage site three nucleotides 5′ to the junction, indicates that it was not cleaved. We assume, therefore, that the endpoint of sequences required for specific cleavage may be close to the LTR terminus. The distal requirements on the U5 side of the junction, identified by comparison of results with clones M13mp18:6(+) and 7(+), is between 17 and 22 nucleotides. Results with clone 6(+) are shown in Fig. 8B.

We conclude from these analyses that the boundary of sequence required for cleavage in the plus strand is not the same as the boundary of sequence required for cleavage in the minus strand.

Comparison of specific cleavage near the U5/U3 LTR junction between pp32 and the αβ form of *pol*-endo. *pol*-derived endonuclease activity is associated with the αβ and ββ forms of reverse transcriptase and with pp32 (4, 7, 12). However, the αβ form requires $MnCl_2$ for activity, whereas the other two forms of the endonuclease are active in the presence of either Mg^{2+} or Mn^{2+}. It was therefore of interest to determine if the site-specific properties of the *pol*-endo are

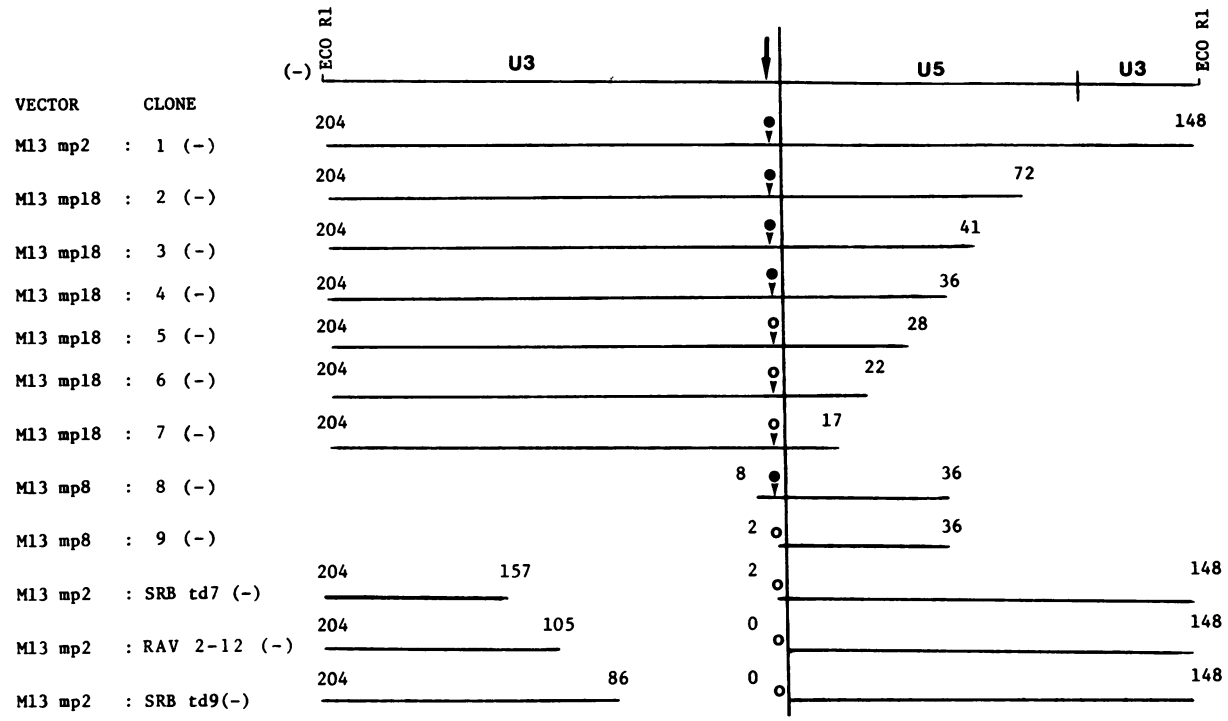

FIG. 5. Limits for sequence recognition and specific cleavage by the *pol*-endo near the U5/U3 junction of the RAV-2 LTR minus strand: summary of experiments with deletion mutants of the permuted RAV-2 LTR. The construction of the BAL 31 deletions of the *Eco*RI-permuted RAV-2 LTR is described in Materials and Methods. The horizontal line at the top represents the *Eco*RI-permuted LTR presented in the minus orientation. Moving left to right along this line corresponds to the 5'-to-3' direction in the DNA strand. The long vertical line marks the unique U5/U3 junction. The short vertical line designates the U3/U5 junction of the LTR. The arrow and arrowheads show the predicted site of cleavage by the *pol*-endo. Closed circles indicate that this site was cleaved while open circles indicate that it was not. Open circles in the absence of an arrowhead indicate that a cryptic site of cleavage occupying the same relative position as the *pol*-endo cleavage site was not observed. Numbering for the endpoints of the recombinant molecules starts with the first nucleotide on either side of the junction and extends into the U3 or U5 regions for the indicated number of bases.

maintained in pp32 and what effects, if any, the divalent cation has upon the choice of cleavage site. The αβ form and pp32 were, therefore, incubated with M13mp2 RAV-2-2(+) in the presence of either Mg^{2+} or Mn^{2+}, and the primer extension assay was carried out as previously described (4). Under these conditions the cleavage site detected in each case was identical (data not shown) and was the same as indicated in Fig. 2. This comparison was repeated with the limit site clone, M13mp19:8(+) (U5, 36/U3, 8) (Fig. 9). The pp32 protein was found to cleave near the U5/U3 junction (lanes 3 and 4) at the same site as the αβ form of *pol*-endo (lane 2), and the site of cleavage was the same with either divalent cation.

DISCUSSION

Our earlier observation that the *pol*-endo from avian retroviruses can selectively cleave the LTR sequence near a region known to be joined to the host during integration strongly suggested a role for its activity in this process (4). This hypothesis has been supported by genetic experiments

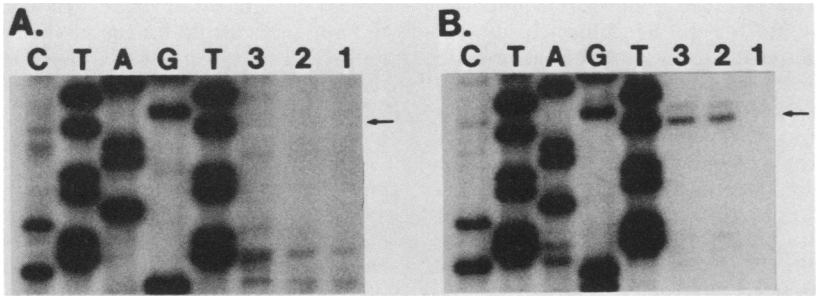

FIG. 6. Mapping of the *pol*-endo cleavage site in the RAV-2-2(−) LTR DNA strand. *pol*-endo cleavage sites in M18mp18:4(−) and M13mp18:5(−) were mapped by the primer extension protocol as described in the legend to Fig. 5 and in Materials and Methods. (A) Results with M13mp18:5; (B) results with M13mp18:4. The endpoints of these clones are shown in Fig. 5. The arrow indicates the predicted site of cleavage by the *pol*-endo. Lane 1, no *pol*-endo; lanes 2 and 3, *pol*-endo incubated with DNA for 20 min.

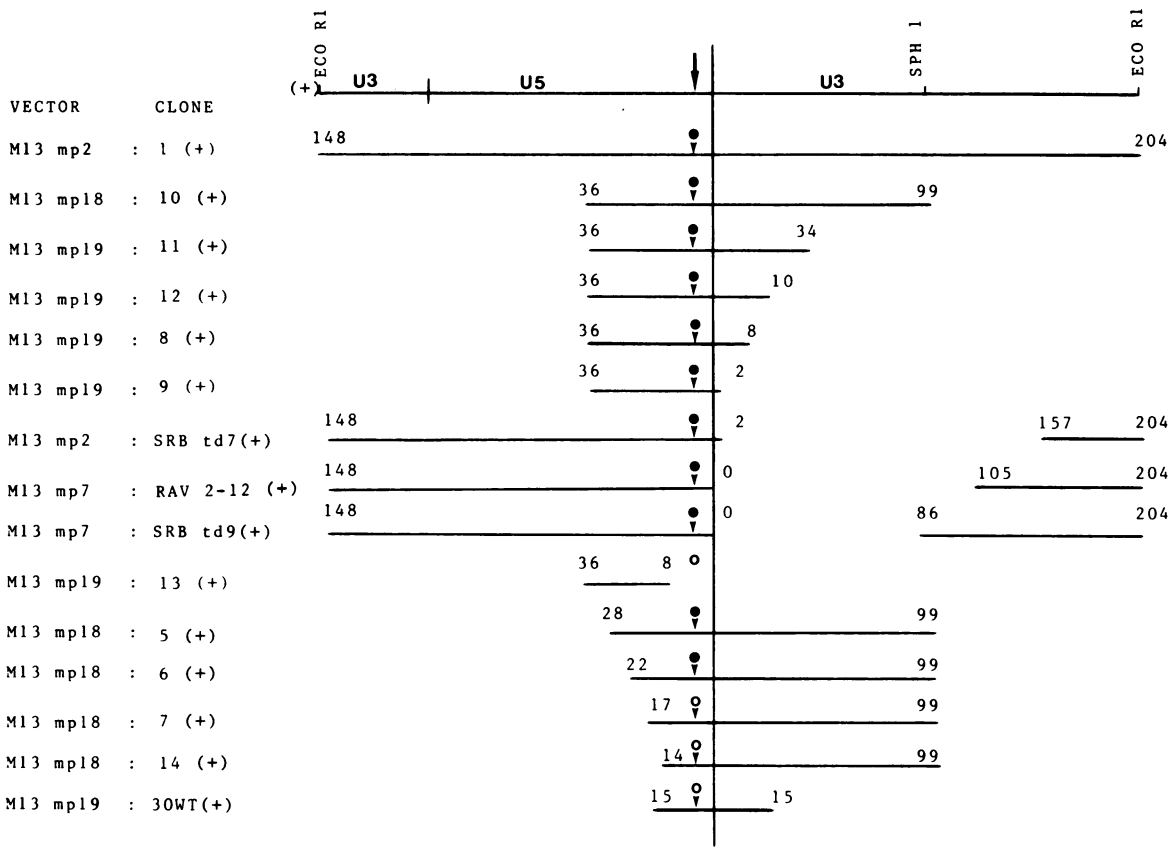

FIG. 7. Limits for sequence recognition and specific cleavage by the *pol*-endo near the U5/U3 junction of the RAV-2 LTR plus strand: summary of experiments with deletion mutants of the permuted RAV-2 LTR. The construction of the BAL 31 deletions of the *Eco*RI-permuted RAV-2 LTR is described in Materials and Methods. The horizontal line at the top represents the plus orientation. Symbols are as in the legend to Fig. 5.

in other retroviral systems which have shown that there is a block to viral DNA integration when mutations are made in the viral DNA in the region of the *pol* gene encoding the endonuclease or at the ends of the LTR which form the U5/U3 junction (3, 17–19, 22). The studies in this report further delineate properties of the *pol*-endo. The αβ and pp32 protein forms have been shown to cleave single-stranded or RF I DNA substrates at the same site near the termini of the RAV-2 LTRs. Thus, all of the specificity for cleavage is maintained in the pp32 domain, independent of the other enzymatic activities which are included in the αβ form. The cleavage sites in both strands of DNA are identical and are found three bases 5′ to the intact U5/U3 junction.

The preference of *pol*-endo for the RF I form of duplex DNA had suggested earlier that the enzyme may recognize single-stranded regions in its substrate (12). This has been verified by in vitro analyses which show that the *pol*-endo cleavage site is the same in RF I and single-stranded DNA substrates. Also, using single-stranded DNA templates, we found that the requirements for specific cleavage are different for each strand (Fig. 10A). The U5/U3 junction domain can be thought of as composed of two subsites, one specifying cleavage on the U5 side of the junction (plus strand) and the other specifying cleavage on the U3 side of the junction (minus strand). Cleavage within the plus strand is independent of sequence information derived from the minus strand and requires 18 to 22 bases 5′ to the intact U5/U3 junction. The information required for cleavage within the minus strand is contained within a region of at most 44 nucleotides in length. The minus-strand domain requires no more than 8 bases 5′ of the intact U5/U3 junction and between 29 and 36 bases 3′ of it. These subsites share some structural features. In both instances, the actual sites of cleavage are within the 12-of-15-base-pair nearly perfect IR, and cleavage at both subsites requires sequences flanking the U5 portion of the IR. These flanking sequences are complementary but are not related in any other obvious manner. Furthermore, the flanking sequences are 5′ of the cleavage site in the plus strand and 3′ of the cleavage site in the minus strand. Considering the fact that these subsites can be recognized in single-stranded DNA, we propose that the *pol*-endo would independently recognize each strand in the RF I provirus. Also, it seems noteworthy that the *pol*-endo recognizes only a part of the palindrome at the U5/U3 junction. Thus, recognition seems to be asymmetric in two dimensions.

Another significant feature of this system is that the *pol*-endos from several different avian viruses, AMV, Rous sarcoma virus (RSV) Pr-C, and RSV B77, recognize and cleave the RAV-2 U5/U3 junction (4). This suggests that the endonucleases from these viruses may be interchangeable with respect to integration. It is consistent with the observation that AMV, which has genetic lesions in the carboxy-terminal domain of *pol*, can be rescued from a nonproducer cell line by infection with RSV B77 *td* and can integrate in a normal manner (25). A comparison of the nucleotide se-

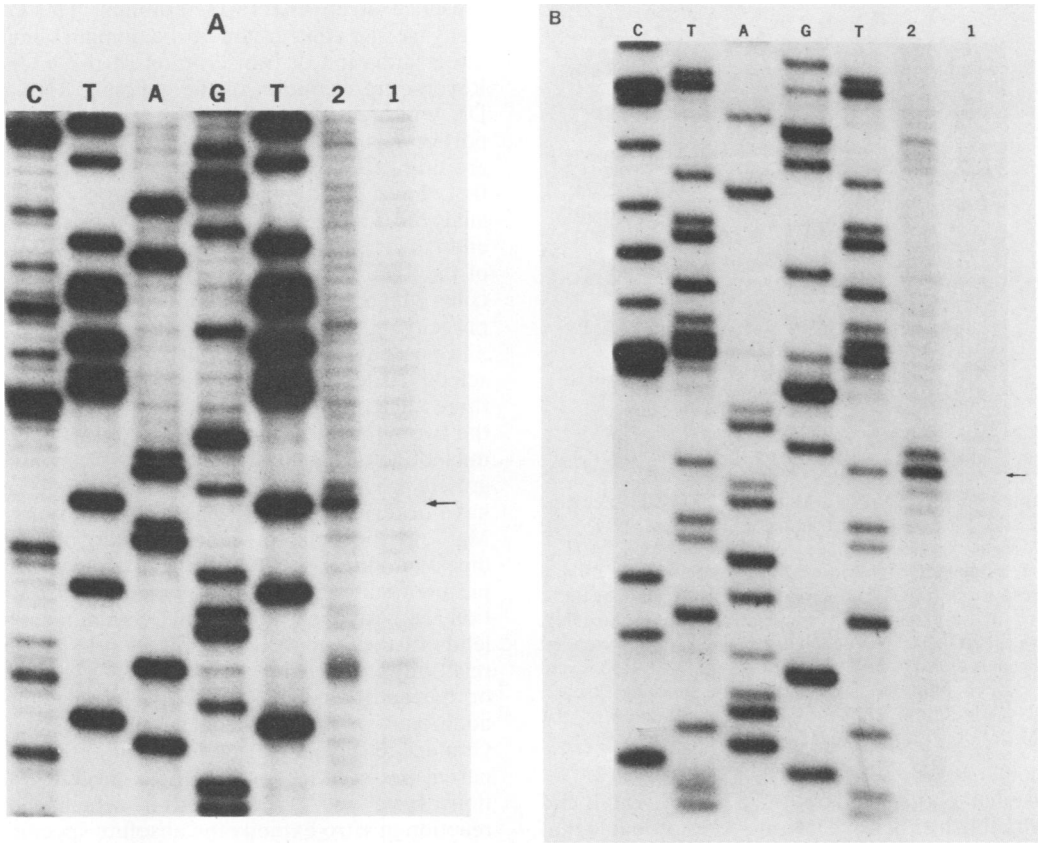

FIG. 8. Mapping of the *pol*-endo cleavage site in SRB *td*-9 (+) and M13mp19:6(+) DNA. (A) The AMV αβ *pol*-endo (9.5 polymerase units) was incubated with 600 ng of SRB *td*-9 (+) DNA in the presence of 2 mM $MnCl_2$, and then primer-extended products were prepared and analyzed as in Materials and Methods. (B) The AMV αβ *pol*-endo (19 polymerase units) was incubated with 1,200 ng of M13mp19:6(+) DNA and analyzed as in panel A. In both panels, material shown in lane 1 was incubated without the AMV αβ *pol*-endo for 20 min, and material shown in lane 2 was incubated with the *pol*-endo for 20 min.

quences of the RAV-2 LTR within the *pol*-endo recognition sequence with analogous sequences in the LTRs of other ASLV retroviruses reveals a high degree of homology (Fig. 10B). There are only a few single-base differences between RAV-2 and the other retroviruses on the U5 side of the junction. Modest sequence variations in this region may be permitted if secondary structure as well as primary sequence is important for recognition. This is consistent with the results of Panganiban and Temin (17), which demonstrate that mutant spleen necrosis virus (SNV) genomes with identical extents of deletions near the LTR termini but with different flanking regions have contrasting biological functions, i.e., they are either capable or incapable of supporting integration. In contrast to the U5 side of the junction where the sequence homology extends beyond the limit of sequences required for specific cleavage, the U3 side is highly divergent. For instance, the RAV-2 and AMV LTR homology is lost after the 12th nucleotide, and the RAV-2 and the *ev*-1 homology is lost after the 9th position. The pattern of sequence conservation on the U3 side of the junction (Fig. 10B) as well as the experimental evidence here predict that a limited number of bases at the junction are required for integration. Consistent with this prediction is the recovery of an avian retrovirus recombinant of RAV-0 and *td* Pr RSV-B, NTRE (27). This virus is almost indistinguishable from its RAV-0 parent except that the extreme 3′ viral RNA end, including the U3 domain, is derived from *td* Pr RSV-B. The new U3 domain diverges from the RAV-0 U3 domain after the ninth position, suggesting that no more than nine bases on the U3 side of the junction are required for integration in vivo.

Whether the DNA fragment containing the *pol*-endo LTR recognition sequence will serve as an integration donor site in vivo is now being tested. It should be noted that cleavage at the junction in DNA clones approximating the size of the limit site is heterogeneous. For example, two primer extension products are observed with the limit sequence clones shown in Fig. 6 and 8 compared with only one in the pdr-R2 DNA shown in Fig. 2C. It will be of interest to determine if a similar relaxation in specificity for integration can be found in vivo by using these limit cleavage site junction regions.

The putative integration site for RAV-2 is structurally similar but not identical to that established for the integration of the SNV (18, 19). Based upon genetic experiments, only 20 bases about the U5/U3 junction of SNV are required for integration. This sequence is also asymmetrically distributed about the U5/U3 junction. However, the predominant contribution is from U3, not from U5, as suggested here for ASLV, and the IR of SNV is 5 nucleotides long compared with 15 for RAV-2. Comparisons of other properties of SNV suggest that this virus may be more closely related to mammalian than to other avian isolates (26). Using the primer extension assay, we failed to detect AMV *pol*-endo cleavage of single-stranded LTR junction sequences of the

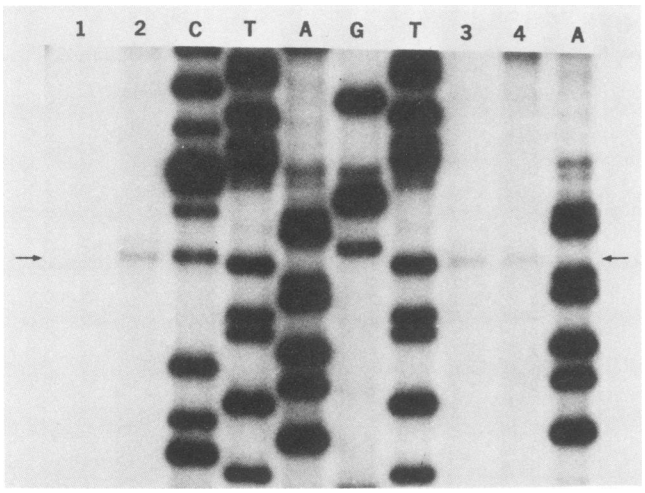

FIG. 9. Mapping of the AMV αβ and pp32 *pol*-endo cleavage sites in RAV-2 LTR (+) DNA strand: M13mp19:8. AMV αβ *pol*-endo or AMV pp32 was incubated with M13mp19:8(+) (U5, 36/U3, 8) (600 ng) as described in Materials and Methods for 20 min at 37°C in the presence of 2 mM $MnCl_2$ or 1 mM $MgCl_2$. Under these conditions, approximately 20% of the single-stranded circular DNA was converted to the linear form in each case. The primer-extended products were prepared and analyzed as described in Materials and Methods. Lanes: 1, incubation of DNA without endonuclease; 2, incubation of DNA for 20 min with AMV αβ *pol*-endo and Mn^{2+}; 3, with pp32 and Mn^{2+}; 4, with pp32 and Mg^{2+}.

Moloney mouse leukemia virus and the SNV (G. Duyk and D. Cobrinik, unpublished data). Thus, it seems probable that there is strict species specificity in the integration machinery of the retroviruses.

The different requirements for cleavage in each strand of ASLV suggest that the continuity of the U5/U3 junction is only required for cleavage in the minus strand. If this observation is relevant to integration it suggests that selection of a candidate for integration will depend upon the presence of an intact U3 terminus. The U3 minus-strand cleavage site is upstream of the domain which is destined to direct initiation of transcription of the integrated provirus. Reverse transcription of the genomic RNA produces some DNA product which is missing all or part of the terminal portion of this U3 domain (10). Such incomplete molecules are often found in the nuclei of infected cells and may, in fact, have given rise to the natural deletion mutants we analyzed in these studies. Thus, the requirement for *pol*-endo to cleave within the nucleotides present at the very end of the upstream LTR ensures selection of only those molecules which are most likely to support efficient production of progeny.

As we have noted previously (4), under our conditions the selective sites of *pol*-endo cleavage at the ASLV junction are three nucleotides removed from the LTR termini instead of the two nucleotides one would expect by comparison of the nucleotide sequences at the host-virus junctions of integrated proviruses. Recently, Grandgenett and co-workers have observed that under certain conditions, which include Mg^{2+} as the divalent cation instead of Mn^{2+} and limited digestion, the pp32 protein will cleave supercoiled DNA preferentially at a site two nucleotides from the LTR junction (D. Grandgenett, personal communication). This result lends further support for a direct role of the endonuclease reaction in viral DNA integration, although more digestion or the addition of Mn^{2+} results in the appearance of an additional six-base-pair staggered cut as reported here (D. Grandgenett, personal communication). Despite these apparent preferences for sites near the LTR junction, conditions have yet to be found in which the endonuclease reaction in vitro exhibits the absolute specificity expected of the integration reaction in vivo. Thus, we suppose that the reaction is more complex than might be predicted by the simplest models (23) and could involve other viral or host proteins. The results of our current studies reinforce that notion. Recognition by the *pol*-endo, which is asymmetric both across the LTR junction and across complementary duplex stands, is surely more complex than first imagined.

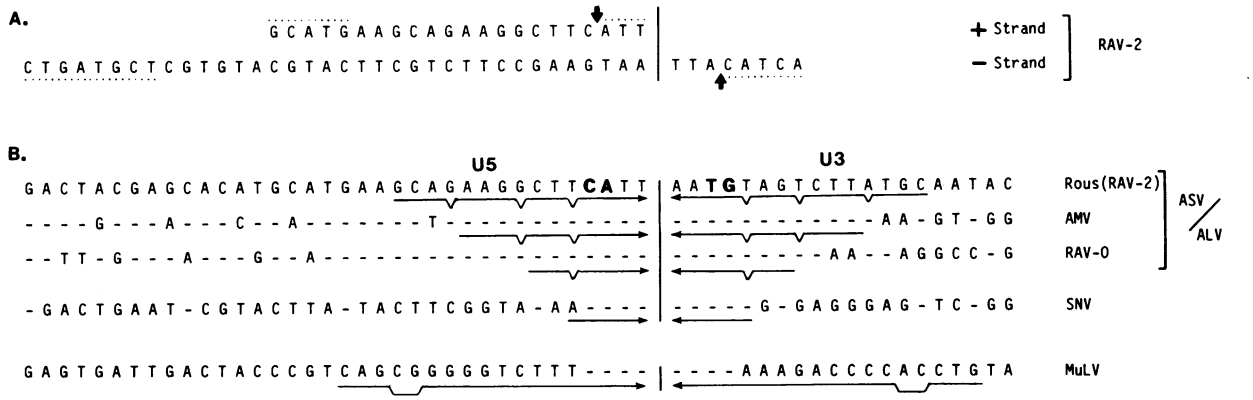

FIG. 10. Comparison of the nucleotide sequences required for site-selective cleavage in the plus and minus strands near the U5/U3 junction of the RAV-2 LTR by the *pol*-endo and the analogous sequences in a number of avian retroviruses. (A) The sequences presented are the inferred limits for sites of recognition and cleavage at the termini of the RAV-2 LTR. This designation is based upon the analysis presented in Fig. 5 and 7. These sites are presumed to represent the upper limit for size of such a domain. The vertical arrow designates the *pol*-endo cleavage site, and the vertical line indicates the position of the U5/U3 junction. The dots under the letters indicate the region of DNA between two DNA clones in which the crossover point between cleavage and no cleavage is observed. (B) Comparison of junction sequences for a number of avian retroviruses. Dashed lines indicate identity; horizontal arrows indicate IRs with breaks sharing noncomplementary bases. Bold-faced letters indicate the termini of viral DNA after integration.

We anticipate that other novel interactions of this protein with its nucleic acid substrates and with other proteins will be discovered.

ACKNOWLEDGMENTS

We are grateful to D. Grandgenett for the gift of the pp32 protein and to J. Messing for M13 vector strains.

This investigation was supported in part by a grant from the American Cancer Society (MV-169) and the National Cancer Institute (CA 38046). G.D. and D.C. are supported in part by a National Institutes of Health Training Grant (T32-GM-07250) from the National Institute of General Medical Science. Both are recipients of a Joseph S. Silber Fellowship from the American Cancer Society, Cuyahoga County Unit. P.D. is supported by Public Health Service grant GM-31808 from the National Institutes of Health.

ADDENDUM IN PROOF

The minimal site clone M13mp19:8(+) has been tested in the RFI form and shown to support site-selective cleavage in both plus and minus strands.

LITERATURE CITED

1. **Caruthers, M., S. Beaucage, Y. Efcavitch, E. Fisher, R. Goldman, P. DeHaseth, W. Mandecki, M. Matteucci, M. Rosendahl, and Y. Stabinsky.** 1983. Chemical synthesis and biological studies on mutated gene-control regions. Cold Spring Harbor Symp. Quant. Biol. **47**:411–418.
2. **deHaseth, P., R. Goldman, C. Cech, and M. Caruthers.** 1983. Chemical synthesis and biochemical reactivity of bacteriophage lambda P_R promoter. Nucleic Acids Res. **11**:773–787.
3. **Donhower, L. A., and H. E. Varmus.** 1984. A mutant murine leukemia virus with a single missense codon in *pol* is defective in a function affecting integration. Proc. Natl. Acad. Sci. USA **81**:6461–6465.
4. **Duyk, G., J. Leis, M. Longiaru, and A. M. Skalka.** 1983. Selective cleavage in the avian retroviral long terminal repeat sequence by the endonuclease associated with the alpha beta form of reverse transcriptase. Proc. Natl. Acad. Sci. USA **80**:6745–6749.
5. **Gibson, W., and I. M. Verma.** 1974. Studies on the reverse transcriptase of RNA tumor viruses: structural relatedness of two subunits of avian RNA tumor viruses. Proc. Natl. Acad. Sci. USA **71**:4991–4994.
6. **Golomb, M., and D. Grandgenett.** 1979. Endonuclease activity of purified RNA-directed DNA polymerase from avian myeloblastosis virus. J. Biol. Chem. **254**:1606–1613.
7. **Golomb, M., D. Grandgenett, and W. Mason.** 1981. Virus-coded DNA endonuclease from avian retrovirus. J. Virol. **38**:548–555.
8. **Grandgenett, D., R. Knaus, and P. Hipponmeyer.** 1983. Antibodies against a synthetic peptide of the avian retrovirus pp32 protein and the β DNA polymerase subunit. Virology **130**:257–262.
9. **Griffith, J., and A. Kornberg.** 1974. Mini M13 bacteriophage: circular fragments of M13 DNA are replicated and packaged during normal infections. Virology **59**:139–146.
10. **Ju, G., L. Boone, and A. Skalka.** 1980. Isolation and characterization of recombinant DNA clones of avian retroviruses: size heterogeneity and instability of the direct repeat. J. Virol. **33**:1026–1033.
11. **Ju, G., and A. M. Skalka.** 1980. Nucleotide sequence analysis of the long terminal repeat (LTR) of avian retroviruses: structural similarities with transposable elements. Cell **22**:379–386.
12. **Leis, J., G. Duyk, S. Johnson, M. Longiaru, and A. Skalka.** 1983. Mechanism of action of the endonuclease associated with the αβ and ββ forms of avian RNA tumor virus reverse transcriptase. J. Virol. **45**:727–739.
13. **Luciw, P., J. Bishop, H. Varmus, and M. Capecchi.** 1983. Location and function of retroviral and SV 40 sequences that enhance biochemical transformation after microinjection of DNA. Cell **33**:705–716.
14. **Maniatis, T., E. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Maxam, A., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74**:560–564.
16. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101C**:20–90.
17. **Panganiban, A. T., and H. M. Temin.** 1983. The terminal nucleotides of retrovirus DNA are required for integration but not for virus production. Nature (London) **306**:155–160.
18. **Panganiban, A. T., and H. M. Temin.** 1984. Circles with two tandem LTRs are precursors to integrated retrovirus DNA. Cell **36**:673–679.
19. **Panganiban, A. T., and H. Temin.** 1984. The retrovirus *pol* gene encodes a product required for DNA integration: identification of a retrovirus *int* locus. Proc. Natl. Acad. Sci. USA **81**:7885–7889.
20. **Rho, H., D. Grandgenett, and M. Green.** 1975. Sequence relatedness between the subunits of AMV reverse transcriptase. J. Biol. Chem. **250**:5278–5280.
21. **Schiff, R., and D. Grandgenett.** 1978. Virus-coded origin of a 32,000-dalton protein from avian retrovirus cores. Structural relatedness of pp32 and beta polypeptide of the avian retrovirus DNA polymerase. J. Virol. **28**:279–291.
22. **Schwartzberg, P., J. Colicelli, and S. P. Goff.** 1984. Construction and analysis of deletion mutations in the *pol* gene of Moloney murine leukemia virus: a new virus function required for establishment of the integrated provirus. Cell **37**:1043–1052.
23. **Shoemaker, C., S. Goff, E. Gilboa, M. Paskind, S. W. Mitra, and D. Baltimore.** 1980. Structure of a cloned circular Moloney murine leukemia virus DNA molecule containing an inverted segment: implications for retrovirus integration. Proc. Natl. Acad. Sci. USA **77**:3932–3936.
24. **Skalka, A. M., G. Duyk, M. Longiaru, P. deHaseth, R. Terry, and J. Leis.** 1984. A role for the avian retroviral reverse transcriptase in the integration of retroviral DNA. Cold Spring Harbor Symp. Quant. Biol. **49**:651–659.
25. **Souza, L., and M. Baluda.** 1980. Identification of the avian myeloblastosis virus genome. J. Virol. **36**:317–324.
26. **Teich, N.** 1984. Taxonomy of retrovirus, p. 25–208. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Tsichlis, P., L. Donehower, G. Hager, N. Zeller, R. Malavarca, S. Astrin, and A. M. Skalka.** 1982. Sequence comparison in the crossover region of an oncogenic avian retrovirus recombinant and its nononcogenic parent: genetic regions that control growth rate and oncogenic potential. Mol. Cell. Biol. **2**:1331–1338.
28. **Varmus, H., and R. Swanstrom.** 1982. Replication of retroviruses, p. 369–512. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Wei, C., G. Alianell, G. Bencen, and H. Gray.** 1983. Isolation and comparison of two molecular species of the BAL-31 nuclease from *Alteromonas espejiana* with disinct kinetic properties. J. Biol. Chem. **258**:13506–13512.
30. **Zasloff, M., G. Ginder, and G. Felsenfeld.** 1979. A new method for purification and identification of covalently closed circular DNA molecules. Nucleic Acids Res. **5**:1139–1145.
31. **Zinder, N., and J. Boeke.** 1982. The filamentous phage (Ff) as vectors for recombinant DNA—a review. Gene **19**:1–12.