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Nonspecific Alcoholysis, a Novel Endonuclease Activity of Human Immunodeficiency Virus Type 1 and Other Retroviral Integrases

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Retroviral integrase (IN) exhibits a previously unrecognized endonuclease activity which we have termed nonspecific alcoholysis. This action occurred at every position in nonviral DNA sequences except those near 5′ ends and is clearly distinguished from, and was not predicted by, the site-specific alcoholysis activity previously described for IN at the processing site near viral DNA termini. The integrases of human immunodeficiency virus type 1, visna virus, and Rous sarcoma virus exhibited different target site preferences in this new assay. The isolated central domain of human immunodeficiency virus type 1 IN preferred the same sites as the full-length protein. Nonspecific alcoholysis may provide insights into the structure and function of IN and other endonucleases and suggests that stimulators of some activities possessed by retroviral enzymes should be sought as antiviral agents.

Retroviral integrase (IN) catalyzes two in vivo endonuclease events that are distinct in specificity, time, and location. After reverse transcription yields blunt-ended linear viral DNA, IN places site-specific nicks adjacent to the invariant CA typically found two nucleotides from the 3′ end of each DNA strand to produce recessed 3′-OH ends; this endonuclease action can occur in the cytoplasm of an infected cell (2, 15). Later, within the cell nucleus, IN inserts the recessed 3′ ends of viral DNA into each strand of host DNA at staggered cleavage sites (the second endonuclease event) that exhibit no sequence consensus but are separated by a small, fixed number of base pairs. Subsequent repair in vivo results in an integrated provirus with a characteristic loss of two base pairs from each end and a short duplication of flanking host DNA (17, 27, 44).

These two actions of IN are modeled in vitro using duplex oligonucleotide substrates designed to mimic the viral DNA termini (22). For example, duplex 18-mers representing one terminus of human immunodeficiency virus type 1 (HIV-1) DNA can serve as substrates for purified HIV-1 IN (Fig. 1A). Site-specific nicking (or processing) by IN at the position following the CA yields DNA products shortened by two nucleotides (Fig. 1B, arrow). The nucleophile utilized for this precise endonuclease event typically is an oxygen atom from water, but OH groups of a variety of alcohols also can be used by IN for what, in essence, is a site-specific alcoholysis reaction (43). IN also catalyzes insertion of processed viral DNA termini into various sites along other oligonucleotides that serve as surrogates for host DNA (7, 20). This coupled cleavage and DNA joining (or strand transfer) reaction yields a set of products that migrates more slowly than the substrate (Fig. 1B, brackets). These bands are detected more easily with longer radiographic exposures (Fig. 1C, brackets).

We now call attention to shorter products of this system. Careful analysis of the standard oligonucleotide assay often reveals rapidly migrating bands that reflect secondary cleavages at positions other than the biologically relevant processing site. These additional bands (seen faintly in the lower half of Fig. 1B, lane 3, and more obviously in Fig. 1C, lane 2) form a distinct pattern that is a function of the sequence of the DNA substrate and the viral source of IN (25). These products cannot all be attributed to insertion of processed viral DNA ends into the 50% of DNA strands that are 5′ labeled (Fig. 1A), as has been presumed (7, 20, 24, 42), because the sum of the intensities of these bands often exceeds 50% of the intensities of the longer strand transfer products (e.g., Fig. 1C, lane 2). Investigation of these bands revealed a previously unrecognized in vitro activity of retroviral integrases.

Glycerol stimulates IN-mediated DNA cleavage at many sites. HIV-1_{HXB2} IN with an N-terminal hexahistidine extension was purified under native conditions from a bacterial expression system (QIAGEN, Inc., Chatsworth, Calif.) as described previously (25). Oligonucleotides were 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Promega Corp., Madison, Wis.), gel purified, and annealed to their complementary sequence (25). Standard 10-μl reaction mixtures contained 0.04 to 0.5 pmol of double-stranded DNA, 25 mM Tris-HCl (pH 8.0), 10 mM dithiothreitol, 10 mM $MnCl_2$, and 0.5 to 1.0 μl of IN or protein buffer (25). Following incubation at 37°C, reactions were stopped, aliquots were electrophoresed on denaturing 20% polyacrylamide gels, and gels were autoradiographed at −80°C (25). When glycerol was added to standard reactions with HIV-1 IN to try to increase strand transfer, as described for one study using Moloney murine leukemia virus IN (8), we noted that some shorter products were enhanced instead (Fig. 1C). Increasing concentrations of glycerol stimulated formation of faster-migrating bands at positions 4 to 14, with maximal effect observed at 40% glycerol. Cleavages occurred at every site except those close to the 5′ end of the DNA, but reproducible preferences were noted for positions 7, 8, and 14 nucleotides from that end. In contrast, glycerol did not stimulate production of the site-specific 16-mer cleavage product or longer strand transfer products, and high glycerol concentrations often inhibited both of these reactions. Shorter

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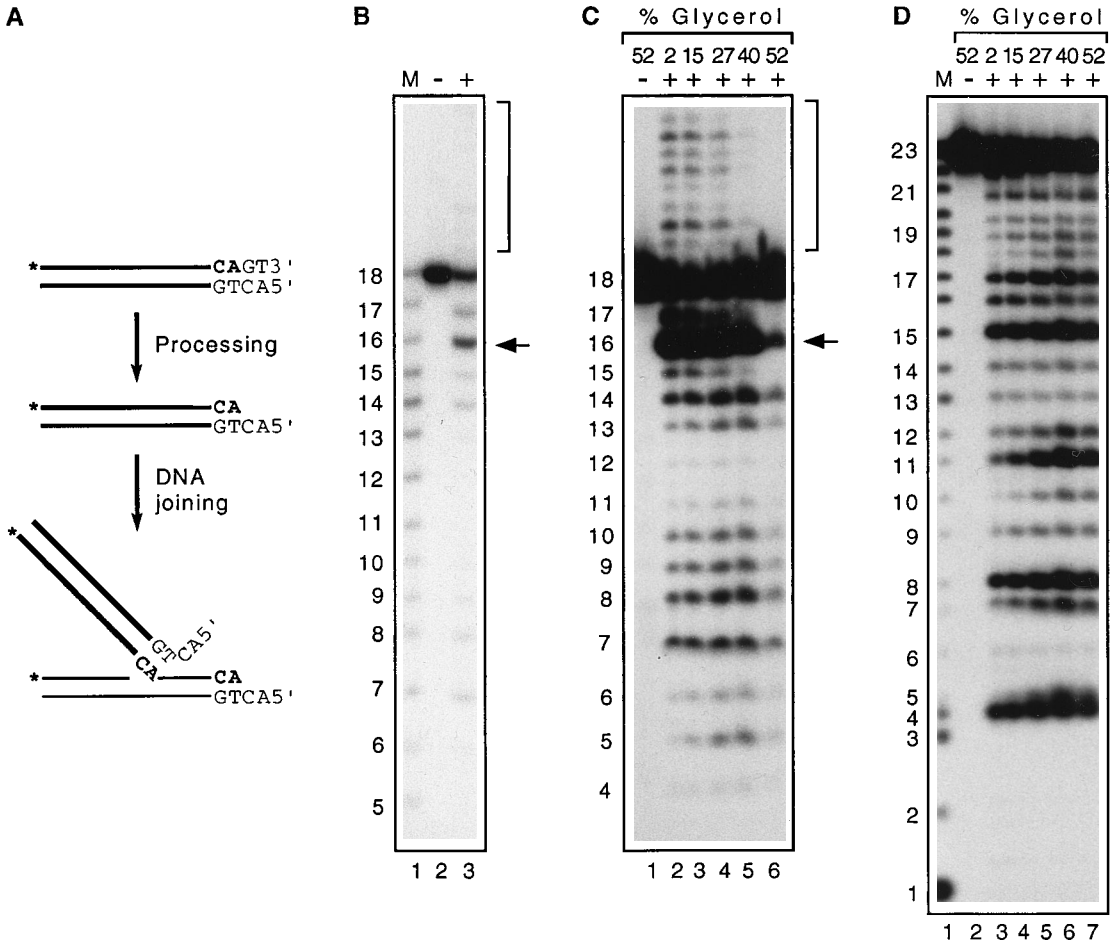

FIG. 1. In vitro integrase assays. (A) Schematic of the standard assay. Duplex oligonucleotides that mimic one end of unintegrated retroviral DNA are 5′ end radiolabeled (asterisk) on the strand that contains the conserved CA (boldface). The processing activity of IN places a specific endonucleolytic nick after the CA, yielding a labeled oligomer two nucleotides shorter than substrate. The DNA joining or strand transfer activity of IN inserts the recessed . . .CA3′-OH terminus into various sites along other oligonucleotides (shown as thin lines) to create a set of labeled integration products longer than substrate. The target DNA strand may or may not be processed or labeled. Processing and DNA joining can occur sequentially in one assay. IN also can catalyze the reverse of strand transfer, termed disintegration. (B) Autoradiogram of denaturing polyacrylamide gel analysis of the integrase assay. Oligonucleotides derived from the HIV-1 U3 terminus (the minus strand sequence is 5′TGAATTAGCCCTTCCAGT3′) served as substrates. Incubation was for 60 min with protein storage buffer (-; lane 2) or purified HIV-1 IN (+; lane 3). The arrow denotes the specific 16-mer cleavage product, and the bracket indicates the position of strand transfer products. Lane 1 contains sequence-specific oligonucleotide markers (M); lengths in nucleotides are indicated at the left. (C) Effect of glycerol on the standard assay. Reactions were conducted for 30 min in the presence of increasing concentrations of glycerol; amounts shown include 2% glycerol provided by the enzyme buffer. Notations are as in panel B. No major bands were detected in reaction lanes below the positions of 4-mers. (D) Glycerol-stimulated cleavage in the absence of viral DNA. A 23-mer of nonviral sequence (5′GAGACTACGT TCGAGGATCCGAG3′) was 5′ end labeled, annealed to its complement, and used as substrate during 60-min reactions. Notations are as in panels B and C.

products did not form in the presence of extra glycerol when IN was omitted (Fig. 1C, lane 1). Similar results were observed when we used preprocessed substrates with 3′ recessed ends (21). The dissociation of the amounts of strand transfer and shorter products suggested that many of the latter result from cleavages that utilize a nucleophile other than processed viral DNA termini.

To verify that IN can utilize nucleophiles other than viral DNA ends, a 5′-labeled 23-mer of nonviral sequence was tested as a substrate for HIV-1 IN (Fig. 1D). As before, increasing glycerol concentrations increased the yield of cleaved products. Cuts occurred at every position in the DNA substrate except those close to the 5′ end and thus can be considered nonspecific. However, preferences were consistently exhibited for positions 4, 5, 8, 11, 15, and 17, suggesting that the reproducible pattern of short products observed in the standard assay with viral DNA substrates results from a similar mechanism. Several lines of evidence support this activity being intrinsic to IN. Neither parallel fractions of a mock purification from the bacterial expression system nor purified HIV-1 IN with Ile substituted for Asp at active-site residue 116 exhibited this activity (21). In addition, HIV-1 IN purified from bacterial inclusion bodies under denaturing conditions and then allowed to refold exhibited a similar pattern of preferred cleavage sites in the presence of extra glycerol (21). Moreover, contaminating nucleases or nonspecific degradation during incubation (3, 19) cannot explain the distinct cleavage patterns created by different integrases purified from the same bacterial expression system (25). Finally, Rous sarcoma virus (RSV) IN purified from bacteria by using a different expression system and avian myeloblastosis virus IN purified from virions had similar activities (21, 39). Since no viral DNA sequences were present in the experiment shown in Fig. 1D, an alternative nucleophile must have been used by IN.

Hydrolysis does not explain these results. Analysis of the new ends of cleavage products indicated that water did not

provide the nucleophile for cutting the DNA phosphodiester bonds. Products 5′ of the cleavage sites did terminate with 3′-OH groups since they comigrated (Fig. 2A, lanes 1 and 2) with sequence-specific 5′-end-labeled oligonucleotide markers produced by the 3′-to-5′ exonuclease activity of snake venom phosphodiesterase (Sigma Chemical Co., St. Louis, Mo.) (22). However, products 3′ of the cleavage sites could not begin with unmodified 5′-P groups because analogous bands formed from a nonviral DNA substrate that was labeled near the 3′ end did not comigrate with a DNase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) ladder (43) created from the same DNA (Fig. 2A, lanes 4 and 5; one of several new bands in lane 4 is indicated with an arrow). The 3′-end-labeled substrate was prepared by extension of the 23-mer by using [α-^{32}P]dCTP, an appropriate 24-mer template, and the Klenow fragment of DNA polymerase I (Promega) (24).

To analyze the nature of the new ends of cleaved products enzymatically, a sample from a completed reaction that had utilized DNA labeled near the 3′ end was heated to 95°C for 5 min to inactivate IN. Aliquots of the reaction sample were treated with calf intestine alkaline phosphatase (Boehringer Mannheim) in 10 mM Tris-HCl (pH 8.3), 1 mM $MgCl_2$, and 1 mM $ZnCl_2$ for 15 min at 37°C and 45 min at 55°C (24) or with T4 polynucleotide kinase and 1 mM ATP for 45 min at 37°C. Reactions were adjusted to similar buffer conditions before gel analysis. The novel bands created by HIV-1 IN in the presence of extra glycerol were not affected by treatment with alkaline phosphatase (for example, compare the bands indicated by the arrow in lanes 3 and 4 of Fig. 2B), confirming that these products do not have unmodified 5′-P groups. An internal positive control for the phosphatase treatment was provided by the small amounts of 3′-end-labeled cleavage products that did migrate with the DNase I ladder in some experiments. These bands reflect a low level of hydrolysis that occurred both spontaneously (Fig. 2B, lane 2) and during incubation with IN (not seen in Fig. 2A, lane 4, but observed in Fig. 3, lanes 3, 8, and 13). The intensities of these bands were markedly diminished after phosphatase treatment with the concurrent appearance of more slowly migrating bands, consistent with removal of the nonradioactive 5′-P group from the hydrolysis products (for example, the band at the position of 8-mers in Fig. 2B, lane 3, is not detected in lane 4, but a new band between positions 9 and 10 is seen in lane 4). Similar results were found when shrimp alkaline phosphatase was used (21). Free 5′-OH groups on the products released 3′ to the site of cleavage also were excluded when T4 polynucleotide kinase was unable to phosphorylate these products efficiently (11), although the full-length oligonucleotide substrate was modified by this treatment and migrated through the gel more quickly (compare lanes 3 and 5 of Fig. 2B). Thus, simple hydrolysis to either side of the phosphodiester DNA linkages cannot account for the novel products produced by IN in the presence of glycerol.

IN mediates nonspecific alcoholysis of nonviral DNA. If glycerol-stimulated, IN-mediated DNA cleavage can utilize a nucleophile other than viral DNA ends or water, what is the nature of the nucleophile utilized for these reactions? Following the logic employed by Vink et al. (43) when they demonstrated that IN catalyzes site-specific alcoholysis of viral DNA termini, we used different alcohols to determine whether glycerol merely stimulated nonspecific cleavage or actually participated in the reaction. For these experiments, HIV-1 IN was purified in the absence of glycerol and assayed immediately on duplex 24-mers of nonviral sequence labeled near the 3′ end of one strand. The alcohols 1,2-ethanediol (ethylene glycol; Fig. 3, lanes 2 to 6), 1,2-propanediol (propylene glycol; lanes 7 to

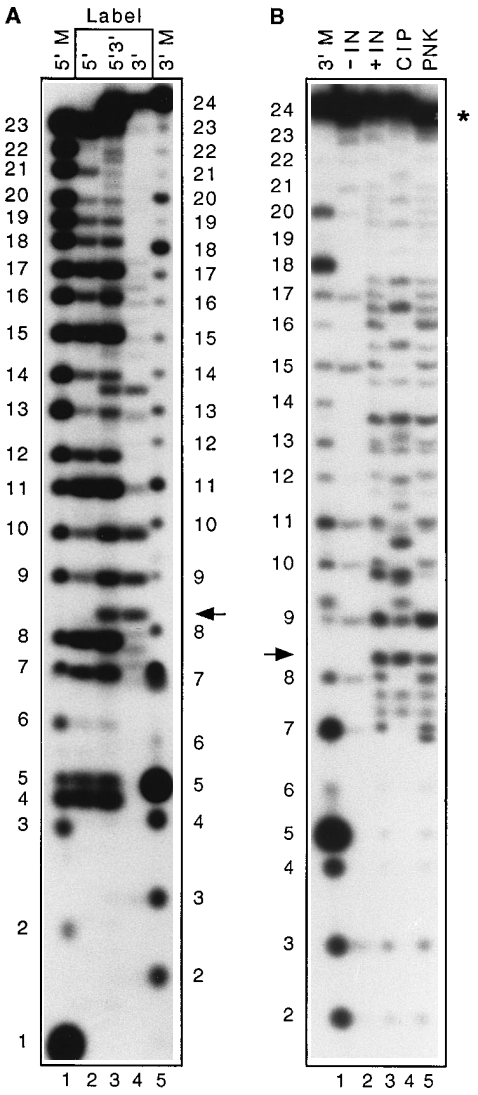

FIG. 2. Analysis of new ends of cleaved products. (A) Comparison with alternatively labeled markers. Duplex 23-mers (lane 2) or 24-mers (lanes 3 and 4) of nonviral sequence were used as substrates for HIV-1 IN during 90-min incubations in the presence of 42% glycerol. One strand of each substrate was 5′ end labeled (lane 2), 5′ and 3′ end labeled (lane 3), or 3′ end labeled (lane 4). Products in lane 2 comigrate with a snake venom phosphodiesterase ladder produced from the 5′-end-labeled 23-mers (5′M; lane 1), indicating the presence of 3′-OH groups. Bands in lane 4 (one is indicated by the arrow) do not align with a DNase I ladder produced from the 3′-end-labeled 24-mers (3′M; lane 5), excluding unmodified 5′-P groups. Marker lengths in nucleotides are indicated to the sides. As expected, lane 3 exhibits the bands of lanes 2 and 4. (B) Enzymatic analysis of new ends. Nonviral duplex 24-mers labeled near the 3′ end of one strand were incubated for 120 min with 34% glycerol and protein buffer (lane 2) or HIV-1 IN (lane 3). Before gel analysis, aliquots of the latter reaction were treated with calf intestine alkaline phosphatase (CIP; lane 4) or polynucleotide kinase (PNK; lane 5). Lane 1 (3′M) contains a DNase I ladder produced from the labeled strand, and lengths in nucleotides are indicated at the left (the anomalous band between positions 9 and 10 was not seen with other preparations and likely results from loss of a nonradioactive 5′-P from some 8-mers). All of the novel products in lane 3 were resistant to CIP (one is indicated by the arrow), confirming the absence of unmodified 5′-P groups. Hydrolysis products in lane 3 were sensitive to CIP and migrated more slowly (note disappearance of bands at marker positions and appearance of new bands; migration was most altered for shorter oligomers). Resistance of most bands to PNK indicates absence of 5′-OH groups. Exceptions are the original 3′-end-labeled 24-mer, which migrated faster after phosphorylation (the asterisk), and the band near position 10 in lane 3, which diminished in intensity, while a new band appeared near position 7 in lane 5 (similar actions of PNK on some alcoholysis products have been described by others [11]).

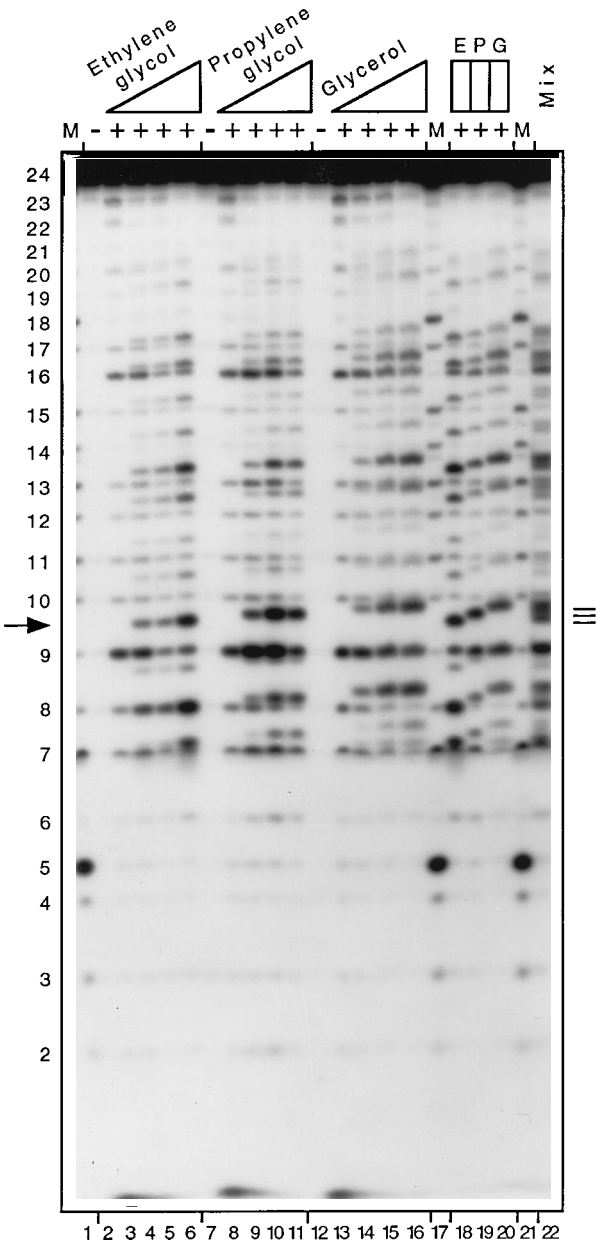

FIG. 3. Effects of different alcohols on IN-mediated cleavage of 3′-end-labeled nonviral DNA. Glycerol-free HIV-1 IN was assayed for 60 min on duplex 24-mers of nonviral sequence that had been labeled near the 3′ end of one strand. Each set of reaction mixtures incubated with IN (+ lanes) contained 0, 15, 30, or 45%, respectively, ethylene glycol (lanes 3 to 6), propylene glycol (lanes 8 to 11), or glycerol (lanes 13 to 16). Reaction mixtures in lanes 2, 7, and 12 were incubated with protein buffer and 30% the indicated alcohol. Samples for lanes 6, 11, and 16 also were loaded in lanes 18 (ethylene glycol [E]), 19 (propylene glycol [P]), and 20 (glycerol [G]), respectively, and aliquots of all three reactions were mixed and loaded in lane 22. Lanes 1, 17, and 21 (marker [M]) contain a DNase I ladder produced from the labeled strand; sizes in nucleotides are indicated at the left. The arrow denotes one of many novel bands that did not migrate with the markers; positions of these bands differed for the three alcohols as indicated for one set at the right.

11), and glycerol (lanes 12 to 16) each stimulated production of at least 15 bands between positions 7 and 21 that did not comigrate with the DNase I ladder (lanes 1, 17, and 21). However, migration of these novel products differed for each of the alcohols, as clearly seen when reactions with the highest alcohol concentrations are examined in adjacent lanes (Fig. 3, lanes 18 to 20). This result was not a gel artifact since mixture of completed reactions did not affect migration of the products (Fig. 3, lane 22). Moreover, the presence of all three alcohols in one reaction led to production of each of the various bands (21). When analyzed on gels with different polyacrylamide percentages, the positions of the novel products relative to the oligonucleotide markers differed (21), suggesting that they contained components other than nucleic acid (43). Some other products did form in the absence of alcohols (Fig. 3, lanes 3, 8, and 13), but these bands comigrated with the markers and thus reflect hydrolysis by IN (dependence on IN is shown by comparison with lanes 2, 7, and 12). These bands tended to diminish as the concentration of each alcohol was increased; this finding is most readily appreciated for the monomer at the bottom of the lanes since some alcohol-dependent bands obscured hydrolysis products.

The results presented above indicate that the three alcohols participated in IN-catalyzed alcoholysis by acting as nucleophiles that became joined to 5′-P groups at sites of DNA cleavage. Thus, IN can utilize these alcohols for nonspecific as well as site-specific alcoholysis (43). 1,3-Propanediol was also able to participate in nonspecific alcoholysis but to a lesser extent (21); this compound reportedly did not support site-specific alcoholysis by IN (43). It is likely that apparent differences in the range of alcohols utilized as nucleophiles for these two activities of IN are more quantitative than qualitative. How IN selects any alcohol molecules, even at the 4 to 6 M concentrations reached in these experiments, rather than the more prevalent water molecules remains an interesting question.

Other integrases and the central domain of HIV-1 IN exhibit nonspecific alcoholysis. We have obtained results similar to those shown in Fig. 3 (21) with visna virus IN purified from the bacterial expression system used for HIV-1 IN (24, 25) and RSV IN purified in an unaltered form by using a different expression system (39). Thus, nonspecific alcoholysis appears to be a general property of retroviral integrases. However, the patterns of preferred cleavage sites differed markedly for the different integrases (Fig. 4A). Although the extent of cleavage varied slightly between repeated assays, the patterns did not. All three integrases were active for nonspecific alcoholysis with Mn^{2+} as a divalent cation, but only RSV IN retained any activity with Mg^{2+}. These results parallel the divalent cation requirements for processing activity by our enzyme preparations (21). Addition of 5% polyethylene glycol, a maneuver reported to promote HIV-1 IN activity with Mg^{2+} (10), did not change the results. The pattern of preferred cleavage sites observed for RSV IN was the same with either divalent cation (21). The isolated central domain of HIV-1 IN, consisting of amino acid positions 50 to 212 (extended by three additional N-terminal amino acids [18] and confirmed to have the expected size by protein gel analysis) was also competent to mediate nonspecific alcoholysis. The pattern of target site preferences observed with this protein fragment, which encompasses the catalytic site of the enzyme, was the same as that seen with the full-length protein (Fig. 4B). This result confirms our previous finding, obtained by analysis of functional chimeric proteins between HIV-1 and visna virus integrases, that the pattern of target site preferences for nonspecific alcoholysis maps to the central region of IN (25). The central domain of RSV IN also has been noted to be responsible for selection of nonspecific cleavage sites on terminal viral DNA sequences (26).

DNA sequence is a major determinant of site preferences for nonspecific alcoholysis. Since it is likely that IN utilizes a single

active site for each of the phosphoryl or polynucleotidyl transfer reactions that it catalyzes (33), understanding the rules by which IN selects preferred DNA sites for any of its actions would be useful. To examine the contributions of base sequence and distance from DNA ends to the preferential susceptibility of certain sites to alcoholysis by IN, we tested the different integrases on a longer nonviral DNA substrate. The 39-mer, 5′gagactaGAGACTACGTTCGAGGATCCGAGctac gttcg3′, was designed to repeat the first seven bases of the 23-mer at its 5′ end (the sequence of the 23-mer is in uppercase) and nine bases that include two highly preferred sites for HIV-1 IN at its 3′ end (underlined). Alcoholysis was monitored as cleavage of 5′-end-labeled DNA in the presence of extra glycerol. Each retroviral IN (Fig. 5A), as well as the central fragment of HIV-1 IN (Fig. 5B), exhibited the same site preferences observed with the shorter substrate. In particular, HIV-1 IN cleaved the 39-mer preferentially at positions 11, 15, 18, 22, and 24 nucleotides from the 5′ end; the base sequences at these positions match preferred sites on the 23-mer (Fig. 6). Some sites near the ends of the 23-mer that had been poor targets for alcoholysis became susceptible to different integrases in the longer substrate. For example, HIV-1 IN did not cleave after position 1 or 2 in the 23-mer but did cleave at these sequences when they were located at positions 8 and 9 in the 39-mer (Fig. 6). The effects of duplicating sites in the longer substrate were variable. HIV-1 IN selected positions 4 and 5 in the 23-mer with moderate frequency and positions 4, 5, and 11 (but not 12) in the longer substrate (Fig. 6). Two sites that were preferentially cleaved by HIV-1 IN in the middle of the 23-mer to produce 8- and 11-mer products were not used efficiently when the surrounding sequence was reproduced near the 3′ end of the 39-mer (i.e., 34- and 37-mers were not prominent products). Preferred alcoholysis sites did not show any sequence consensus, and cleavages were noted after all four bases. The position of the radiolabel did not affect cleavage site preferences (Fig. 6). Thus, DNA sequence is a major determinant of susceptibility to nonspecific alcoholysis by IN, within constraints imposed by being located near DNA ends. Interestingly, 5′ ends of DNA also seem to be poor targets for strand transfer by IN (24, 42). Whether the inefficiency of these activities near DNA ends reflects the same constraints that influence the spacing of staggered nicks in host DNA requires further study.

Implications. We and others have previously noted nonspecific nuclease activity by various IN preparations (4, 10, 22–24, 28, 29, 34, 42); the present report reveals one mechanism underlying this activity, i.e., formation of alcohol-DNA adducts. Nonspecific alcoholysis occurred at every nucleotide position in nonviral DNA except those close to the 5′ end and thus is clearly distinguished from, and was not predicted by, the site-specific alcoholysis activity previously described for IN at the processing site near viral DNA termini (11, 43). However, the two activities share the ability to accommodate a diversity of nucleophiles, suggesting considerable flexibility of the active site of the enzyme (8, 11). Proven sources of nucleophilic OH groups for site-specific viral DNA processing include some alcohols, water, the 3′ ends of viral DNA (to yield 3′-5′ cyclic dinucleotide products [11]), and serine or threonine residues of the enzyme itself (shown only for avian myeloblastosis virus IN [23]). Nucleophiles that can be used by IN to attack phosphodiester bonds in nonviral DNA sequences include alcohols during nonspecific alcoholysis (Fig. 3), water during nonspecific hydrolysis (Fig. 3), processed viral DNA termini during strand transfer, and juxtaposed 3′ ends of nonviral DNA during disintegration reactions that resemble a reversal of strand transfer (Fig. 1A) (5). Factors that contribute to the choice of nucleophile during viral DNA processing include the viral source of IN (8, 11, 14, 24, 35, 41), the identity of the divalent cation (32, 35, 40, 43), steric and electrostatic interactions between IN and substrate DNA (32), and determinants in the central region of IN (9, 40). It would be interesting to apply nonspecific alcoholysis assays to altered IN proteins that have single amino acid substitutions near active-site residues Asp-116 or Glu-152 and reportedly prefer the 3′-OH end of viral DNA termini, rather than water or alcohols, during viral DNA processing (9, 40). However, RSV IN reportedly also preferred

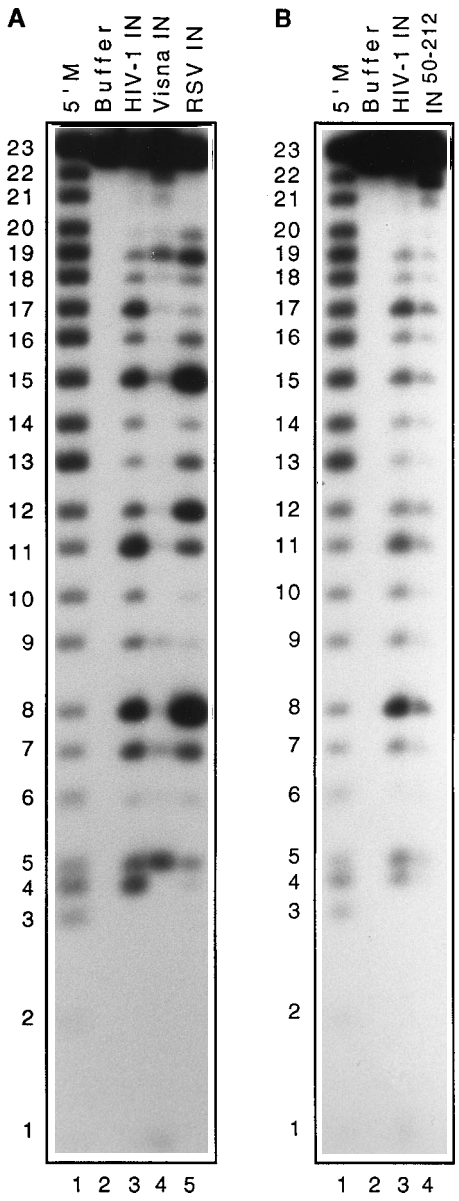

FIG. 4. Alcoholysis patterns produced by different retroviral integrases on 5′-end-labeled nonviral DNA. Duplex 23-mers of nonviral sequence that had been 5′ end labeled on one strand were incubated for 90 min with the indicated proteins in the presence of 44% glycerol. (A) Incubation was with protein buffer (lane 2) or the integrase of HIV-1 (lane 3), visna virus (lane 4), or RSV (lane 5). (B) Incubation was with protein buffer (lane 2), HIV-1 IN (lane 3), or the central domain fragment of HIV-1 IN (IN^{50-212}; lane 4). Lane 1 of each panel contains sequence-specific oligonucleotide markers (5′M); lengths in nucleotides are indicated at the left.

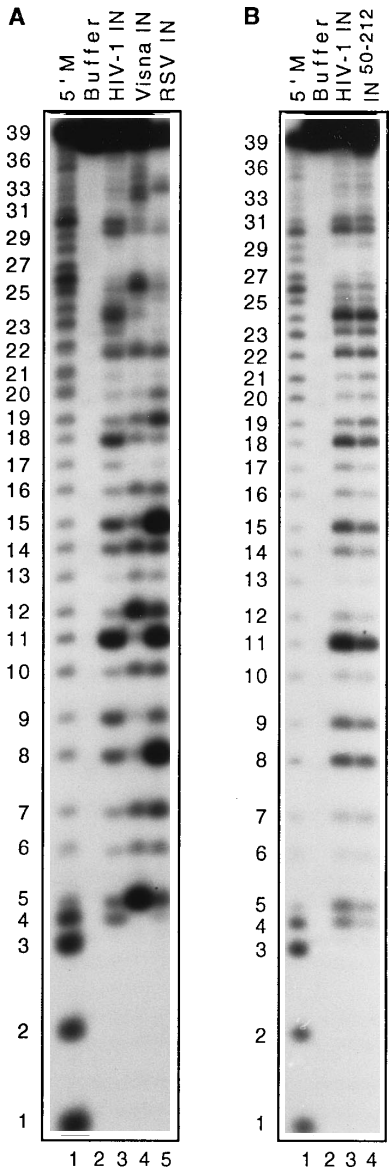

FIG. 5. Alcoholysis patterns on a longer 5′-end-labeled nonviral DNA substrate. Duplex 39-mers of nonviral sequence that had been 5′ end labeled on one strand were incubated for 90 to 120 min with the indicated proteins in the presence of 44% glycerol (A) or 34% glycerol (B). Lanes are as in Fig. 4. Ambiguity in alignment of longer markers between the two panels is due to loading conditions; assignment of nucleotide lengths to cleavage products was made after analysis of multiple gels.

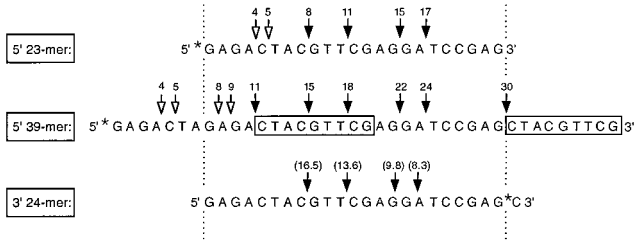

FIG. 6. Schematic of preferential alcoholysis sites for HIV-1 IN on three different nonviral DNA substrates. Prominent cleavage sites for HIV-1 IN on the 5′-end-labeled 23-mer (Fig. 1D and 4) and the 5′-end-labeled 39-mer (Fig. 5) are indicated by closed arrowheads; sites chosen somewhat less frequently are indicated with open arrowheads. Numbers above the arrows correspond to sizes of labeled fragments numbered from the 5′ end. Positions of prominent novel bands for reactions using the 3′-end-labeled 24-mer substrate and glycerol (Fig. 2 and 3) are indicated with closed arrowheads; apparent sizes in parentheses were interpolated from the autoradiograms and are numbered from the 3′ end. Asterisks denote the positions of ^{32}P groups. For comparison, sequences are aligned and the nine bases from the middle of the 23-mer that have been reproduced at the 3′ end of the 39-mer are boxed. As the size of the 3′-end-labeled products increased, the effect of the attached alcohol group on migration through the gel diminished (compare cleavage sites on the upper substrate with locations of corresponding bands from the lower substrate). Similar figures can be produced for visna virus IN or RSV IN.

to make the cyclic dinucleotide product during processing (35) but was very active for nonspecific alcoholysis (Fig. 4 and 5).

Further study of nonspecific alcoholysis may reveal new insights into the structure and organization of IN. This activity is robust; altered integrases that are not active in the standard site-specific processing or DNA joining assays are active in the assay for nonspecific alcoholysis (25). A similar observation holds for in vitro disintegration assays (25). However, the standard disintegration reaction shows little substrate specificity (5, 25, 38) and cannot distinguish between the viral source of different integrases (25). In contrast, the distinctive pattern of preferred sites for nonspecific alcoholysis by different integrases has already provided a powerful tool for mapping functional domains of IN (25). This activity may be particularly well suited for studies aimed at identifying functional nucleophile and target DNA sites on the enzyme. For example, the central domain of HIV-1 IN, which cannot perform viral DNA processing, is competent to mediate nonspecific alcoholysis and thus appears to be involved in selecting the nucleophile for nonspecific attack of host DNA as well as the nucleophile for viral DNA processing (9, 40). Moreover, this domain must position target DNA for nucleophilic attack by different alcohols since it exhibited the same site preferences in this assay as the full-length protein. Alcoholysis assays may provide important clues for understanding how viral DNA termini can act sequentially as target for processing and then as nucleophile for DNA joining.

Is nonspecific alcoholysis a general property of endonucleases? Under certain conditions, RNase A, snake venom phosphodiesterase, and eukaryotic topoisomerase I can mediate alcoholysis in addition to hydrolysis (6, 13, 16). Moreover, high glycerol concentrations induce altered specificity (star activity) of restriction endonucleases; whether nucleophilic groups of glycerol participate in these reactions has not been addressed. However, star activity is induced by many osmolytes, such as ethanol, 2-propanol, or sucrose, that act to remove water from the microenvironment of the enzyme (36, 37). None of these agents (or methanol, 1-butanol, or isoamyl alcohol) stimulated nonspecific cleavage activity by HIV-1 IN (21). In addition, IN-mediated hydrolysis and alcoholysis show similar cleavage site preferences on nonviral DNA, as detected with glycerol-free HIV-1 IN and 5′-end-labeled DNA (21). Thus, unlike the situation with *Eco*RI (36, 37), bound water molecules do not appear to be critical for DNA recognition by IN. The determinants of nucleophile selection as well as DNA specificity for different endonucleases require further investigation.

The biological significance of nonspecific alcoholysis by retroviral IN is unknown, but the diversity of nucleophiles utilized by IN suggests novel therapeutic potential. Unbridling this newly identified nuclease activity might cause IN to destroy recently synthesized viral DNA and abort the infectious process, with minimal toxicity on host DNA if this activity remained sequestered in the viral nucleoprotein complex in the cell cytoplasm. Indeed, murine leukemia virus DNA in such complexes is susceptible to nonspecific digestion by micrococ-

cal nuclease (1). Identification of IN-mediated nonspecific alcoholysis suggests that stimulators, as well as inhibitors, of some activities possessed by retroviral enzymes should be sought as antiviral agents (12, 30, 31).

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