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Human immunodeficiency virus integration protein expressed in *Escherichia coli* possesses selective DNA cleaving activity

(retroviral endonuclease/antiviral target/protein purification)

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ABSTRACT **The human immunodeficiency virus (HIV) integration protein, a potential target for selective antiviral therapy, was expressed in *Escherichia coli*. The purified protein, free of detectable contaminating endonucleases, selectively cleaved double-stranded DNA oligonucleotides that mimic the U3 and the U5 termini of linear HIV DNA. Two nucleotides were removed from the 3′ ends of both the U5 plus strand and the U3 minus strand; in both cases, cleavage was adjacent to a conserved CA dinucleotide. The reaction was metal-ion dependent, with a preference for Mn^{2+} over Mg^{2+}. Reaction selectivity was further demonstrated by the lack of cleavage of an HIV U5 substrate on the complementary (minus) strand, an analogous substrate that mimics the U3 terminus of an avian retrovirus, and an HIV U5 substrate in which the conserved CA dinucleotide was replaced with a TA dinucleotide. Such an integration protein-mediated cleavage reaction is expected to occur as part of the integration event in the retroviral life cycle, in which a double-stranded DNA copy of the viral RNA genome is inserted into the host cell DNA.**

An essential step in the life cycle of retroviruses is insertion of a double-stranded DNA copy of the viral RNA genome into the host cell DNA. The integrated viral DNA, termed the provirus, serves as the template for transcription of viral genes. The insertion event depends on at least one viral protein, the integration protein (IN), which is a product of the viral *pol* gene. Mutations in the IN coding region of *pol* result in integration-negative, replication-defective retroviruses (1–3). The proviral DNA is identical to the precursor viral DNA except for the loss of two base pairs (bp) at each end, at the points of attachment to cellular DNA. Therefore, one proposed function for IN is the removal of two bases from the 3′ termini of both strands of the viral DNA, in preparation for integration.

The IN from avian sarcoma–leukosis viruses (ASLV) has been investigated in detail. A 32-kDa protein, encoded by the genetically defined IN locus, is generated in the virion by proteolytic processing of the 95-kDa β chain of reverse transcriptase (4). This protein selectively binds and cleaves viral DNA at sequences (located in the long terminal repeats) that have been shown to be required for integration (5–7). Avian IN that has been expressed in *Escherichia coli* exhibits enzymatic properties very similar to the protein isolated from virions (8).

The IN from human immunodeficiency virus (HIV) is a potential target for selective antiviral therapy. It, too, has recently been expressed in *E. coli* (9). In this system, crude extracts containing IN exhibited nonspecific DNA binding activity when subjected to protein/DNA blot analysis. Here, we report high-level expression of HIV IN in a different *E. coli* system. Purification of this protein and characterization of its DNA cleaving activity are described.

MATERIALS AND METHODS

Construction of an HIV IN Expression Plasmid. Salient features of the HIV IN expression system are outlined in Fig. 1*A*. Plasmid pART-1 (R. Swanstrom, University of North Carolina at Chapel Hill) contains the HIV IN coding sequence from HXB2D, a functional viral clone (10). A 1663-bp *Kpn* I–*Hin*dIII fragment from pART-1 encompassing the IN gene was subcloned into M13mp19. By means of oligonucleotide-directed mutagenesis, an *Nde* I site was created at the 5′ end of the IN coding sequence. An 898-bp *Nde* I–*Nde* I fragment, which starts at the 5′ end of the IN coding region and ends 27 bp after the IN termination codon was then inserted into plasmid p582 (obtained from S. Short, Wellcome), a derivative of the pET-3C translation vector of Rosenberg *et al.* (11). The ATG in the 5′ *Nde* I site became the translation start codon in the resulting *E. coli* expression plasmid, pT7-IN, in which transcription of the IN gene was under the control of a T7 bacteriophage RNA polymerase promoter. In this system, a single copy of the gene for T7 RNA polymerase is stably incorporated into the chromosome of the host bacterium BL21 (DE3) and is under the control of the *lac* UV5 promoter (12).

Protein Purification. Bacteria that contained either the parental expression plasmid (p582) or the IN expression plasmid (pT7-IN) were grown to an optical density of 1.0 at 600 nm in LB broth supplemented with kanamycin at 50 μg/ml. T7 RNA polymerase was then induced by addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM. Three hours postinduction, bacteria were harvested by centrifugation. Bacteria were resuspended in cold 50 mM Tris·HCl, pH 7.5/5 mM dithiothreitol/1 mM EDTA (6 ml/g of bacteria) and lysed by two passages through a French press. The lysate was centrifuged for 1 hr at 12,000 × *g*. The supernatant was discarded, and the pellet was resuspended in 20 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/1 mM EDTA/1 M NaCl (4 ml/g of bacteria) by using a Dounce homogenizer. The homogenate was gently stirred for 30 min and recentrifuged for 30 min at 30,000 × *g*. The supernatant (designated 1 M NaCl extract) was dialyzed for 2 hr against 0.8 M ammonium sulfate in buffer A (50 mM Tris·HCl, pH 7.5/200 mM NaCl/1 mM dithiothreitol/0.1 mM EDTA). Precipitated material was removed by centrifugation, and the supernatant was loaded onto a butyl-Sepharose 4B (Pharmacia) column (1 mg of protein per ml of butyl-Sepharose) equilibrated with dialysis buffer. An 8- to 10-column-volume rinse was followed by elution with a linear gradient (11 column volumes) of 0.8 M ammonium sulfate in buffer A to 10% (vol/vol) glycerol in buffer A. Fractions that contained IN were diluted

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Abbreviations: HIV, human immunodeficiency virus; IN, integration protein; ASLV, avian sarcoma–leukosis virus; AMV, avian myeloblastosis virus.

with two volumes of 50 mM Tris·HCl, pH 7.5/10% glycerol/1 mM dithiothreitol/0.1 mM EDTA and loaded onto a column of heparin-Sepharose CL-6B (Pharmacia) equilibrated with the same buffer (1 mg of protein per ml of heparin-Sepharose). After washing with several volumes of equilibration buffer, the column was eluted with a linear gradient (10 column volumes) of 0–1 M NaCl.

Endonuclease Assays. Two methods were used to assess DNA cleaving activity. Endonucleolytic cleavage of a heterologous DNA substrate was monitored by observing the conversion of supercoiled (form I) plasmid DNA to nicked (form II) and linear (form III) DNA. Typical reaction mixtures (10 μl) contained 0.2 μg of supercoiled pBR322, 20 mM Tris·HCl (pH 8), 5 mM 2-mercaptoethanol, 2 mM $MnCl_2$, and 1 μl of protein solution. Reactions were incubated for 30 min at 37°C and were stopped by the addition of 1 μl of 0.5 M EDTA plus 2 μl of 15% Ficoll 400 and 0.2% bromophenol blue in TAE buffer (40 mM Tris/20 mM acetic acid/1 mM EDTA). Samples were electrophoresed on 1% agarose gels.

Cleavage of DNA at specific sites in the long terminal repeat sequences of retroviral linear DNA was monitored with double-stranded synthetic oligonucleotides as substrates (sequences are shown in Fig. 5A). Oligonucleotides were prepared with a Biosearch model 8600 DNA synthesizer (W. Dallas, Wellcome) and were purified by electrophoresis on 20% denaturing polyacrylamide gels. The appropriate strands were end-labeled at the 5′ termini with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear). Reaction mixtures (10 μl) for cleaving activity contained ≈10 ng (1 pmol) of end-labeled DNA, 20 mM Tris·HCl (pH 8), 5 mM 2-mercaptoethanol, 1 mM $MgCl_2$ (contributed from the DNA end-labeling reaction), 1 mM $MnCl_2$ as indicated, and 0.2 μg of protein. Incubations were for 1 hr at 37°C and were stopped by adding 15 μl of 90% formamide/0.025% bromophenol blue/0.025% xylene cyanol/89 mM Tris/89 mM boric acid/2 mM EDTA followed by heating for 3 min at 90°C. The samples were electrophoresed on a 20% denaturing polyacrylamide gel. Reaction products were visualized by autoradiography of the wet gel.

DNA Binding Assay. DNA binding activity was assayed by the nitrocellulose filter binding method (13). The end-labeled 20-bp oligonucleotide corresponding to the U5 terminus of HIV retroviral DNA described above for the endonuclease assay was also used for the DNA binding assay. Nitrocellulose filters (25-mm circles, 0.45-μm pore size, Schleicher & Schuell) were soaked in 0.4 M KOH for 40 min, washed in distilled water, and soaked for at least 30 min in binding buffer (20 mM Tris·HCl, pH 7.5/10% glycerol/50 mM KCl/0.1 mM dithiothreitol). Reaction mixtures (50 μl) contained 2 ng of end-labeled DNA, binding buffer, 1 mM $MnCl_2$, and 1 μl of protein solution. Incubations were for 30 min at room temperature. The samples were then slowly filtered through nitrocellulose filters and washed two times with 0.5 ml of binding buffer. Filters were air-dried and counted with an organic scintillation fluid.

Other Methods. SDS/PAGE (12% resolving gel) was performed as described by Laemmli (14). Protein concentrations were determined by the Bradford method (15) with bovine serum albumin as a standard. Western blot analysis was performed with a Bio-Rad mini Trans-Blot electrophoretic transfer cell. The primary antibody was immune serum from an AIDS patient (BB2; provided by R. Swanstrom). Immunoreactive proteins were detected with an alkaline phosphatase-conjugated secondary antibody (Protoblot system, Promega). Analysis of the N-terminal amino acid sequence of IN was performed with an Applied Biosystems model 477a protein sequencer using Edman degradation chemistry with an on-line amino acid analyzer (Applied Biosystems model 120a HPLC) and microcomputer for control and data reduction (W. Chestnut, Wellcome).

RESULTS

Expression of the HIV IN in *E. coli*. The N-terminal amino acid sequence of a 32-kDa putative HIV IN has been determined (16) and, as for other retroviruses, maps to the 3′ end of the *pol* gene. Because IN is a proteolytic cleavage product of a *gag–pol* fusion protein precursor, the gene for IN does not contain an ATG translation start codon. Therefore, an ATG codon was introduced immediately upstream of the codon for the N-terminal phenylalanine in the HIV IN expression plasmid pT7-IN (Fig. 1A). The product of the bacterial expression system was hence designed to be identical to the authentic viral protein, except for an additional N-terminal methionine.

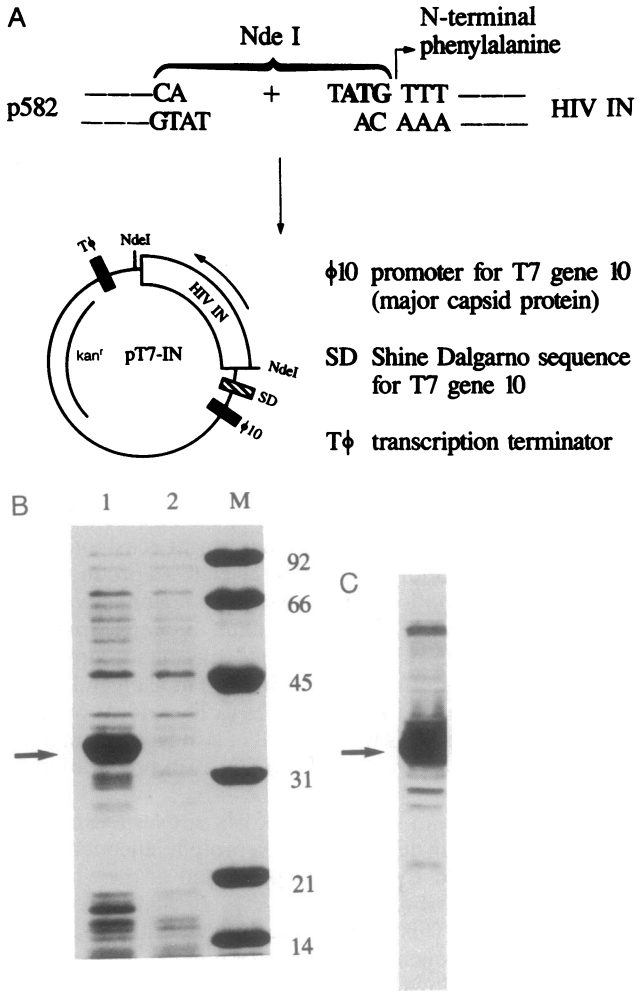

FIG. 1. Expression of the HIV IN in *E. coli*. (*A*) Construction of an HIV IN expression plasmid. The HIV IN gene was placed under the control of a T7 bacteriophage RNA polymerase promoter. The product of the bacterial expression system was designed to be identical to the authentic viral protein, except for an additional N-terminal methionine. (*B*) SDS/PAGE analysis of bacterial expression products. One molar NaCl extracts of bacteria that contained pT7-IN (lane 1) or p582 (lane 2) were analyzed by SDS/PAGE (12% resolving gel). Proteins were stained with Coomassie blue. The arrow marks the position of the 32-kDa HIV IN. Lane M, molecular size markers (in kDa). (*C*) Western blot analysis of a 1 M NaCl extract of bacteria containing pT7-IN. The primary antibody was serum from an AIDS patient. Immunoreactive proteins were detected with an alkaline phosphatase-conjugated secondary antibody.

As described for bacterially produced avian IN (8), the majority of the HIV IN produced in our *E. coli* expression system remained in the pellet fraction following centrifugation of bacterial lysates. A large amount of the protein was released from the pellet by extraction with buffered 1 M NaCl. SDS/PAGE of extracts prepared from bacteria that harbored pT7-IN revealed a protein with a molecular mass of ≈32 kDa, which comprised ≈80% of the extracted protein (Fig. 1*B*). This protein was not observed in extracts prepared from bacteria that contained the parental expression plasmid p582. The 32-kDa protein reacted with serum antibodies from an AIDS patient (Fig. 1*C*) and migrated on SDS/polyacrylamide gels with the same mobility as the putative IN from HIV virions (data not shown). It was concluded that the 32-kDa protein was the product of the HIV IN gene.

DNA Binding and Cleaving Activities Copurify with HIV IN Expressed in *E. coli*. Standard chromatographic methods for the purification of DNA binding proteins including phosphocellulose and heparin-Sepharose failed to resolve HIV IN from endonucleases of bacterial origin. However a DNA cleaving activity (nicking and linearizing of supercoiled plasmid DNA), which coeluted with HIV IN, was resolved from contaminating bacterial endonucleases by butyl-Sepharose chromatography. As shown in Fig. 2, all detectable endonucleases originating from the host bacterial genome (extract of bacteria that contained the parental expression plasmid p582) eluted from butyl-Sepharose well ahead of the 32-kDa HIV IN.

HIV IN was further purified by chromatography on heparin-Sepharose. The protein obtained from heparin-Sepharose was nearly homogeneous and coeluted with DNA cleaving and DNA binding activities (Fig. 3*)*. This protein sedimented slightly ahead of ovalbumin (molecular mass of 45 kDa) on glycerol gradients, suggesting the existence of a dimer under the sedimentation conditions employed (data not shown). Similar behavior on a glycerol gradient was demonstrated for IN from an avian retrovirus (17). Amino acid sequence analysis confirmed the presence of a methionine at the N terminus of the purified protein, and the results for the next seven residues were in agreement with the predicted amino acid sequence (data not shown).

Characteristics of the DNA Cleaving Activity of Purified HIV IN. The DNA cleaving activities of avian myeloblastosis virus (AMV) IN (D. Grandgenett, Saint Louis University Medical Center) and purified HIV IN were compared with supercoiled plasmid pBR322 as a substrate. As shown in Fig. 4, the plasmid-cleaving activities of the two proteins were very similar. In the presence of Mn^{2+}, both proteins converted supercoiled plasmid DNA to nicked and linear forms.

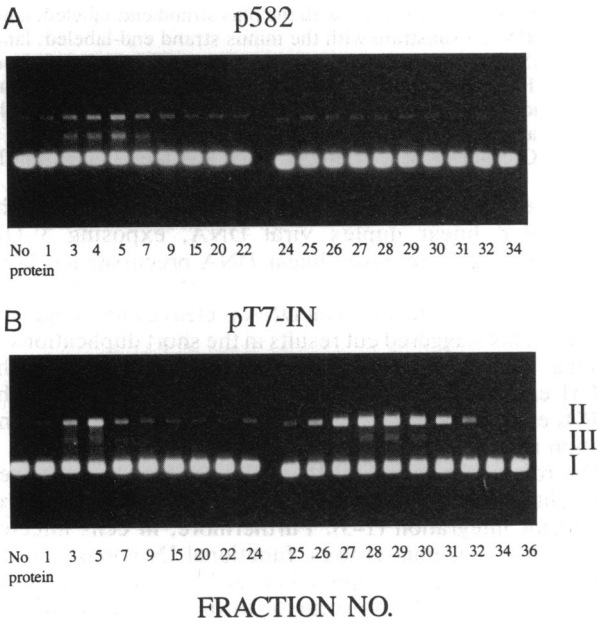

FIG. 2. Resolution of HIV IN from host bacterial endonucleases by chromatography on butyl-Sepharose. (*A*) Endonucleolytic activity of fractions from butyl-Sepharose chromatography of a 1 M NaCl extract of bacteria that contained the parental expression plasmid p582. (*B*) Same as *A*, except that the bacteria contained pT7-IN. Fractions 26–32 contained the 32-kDa HIV IN protein, as determined by SDS/PAGE (data not shown). I, supercoiled; II, nicked; III, linear. The p582 and pT7-IN extracts were from equivalent amounts of bacteria.

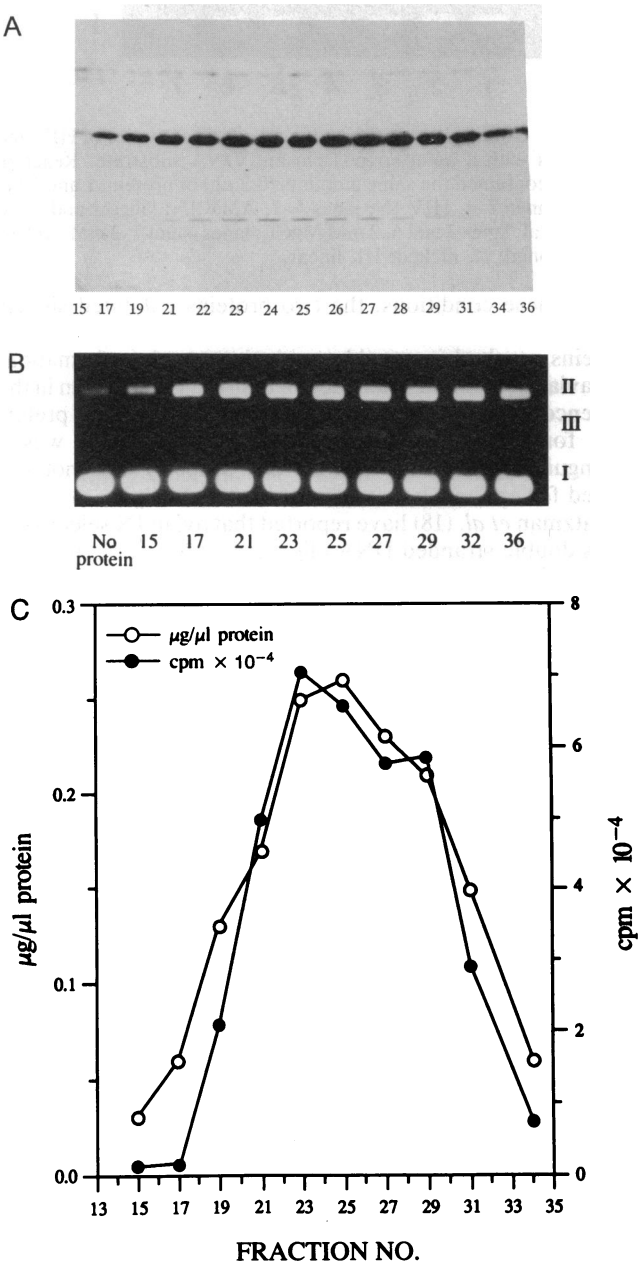

FIG. 3. Heparin-Sepharose chromatography of HIV IN. (*A*) SDS/PAGE analysis; 7.5 μl of column fraction per lane. (*B*) Endonucleolytic activity. Plasmid pBR322 was the substrate. Reactions contained 1 μl of the column fractions. I, supercoiled; II, nicked; III, linear. (*C*) DNA binding and protein assay. A double-stranded oligonucleotide that mimics the U5 terminus of HIV DNA was the substrate in the binding assay. Binding reactions contained 1 μl of the column fractions.

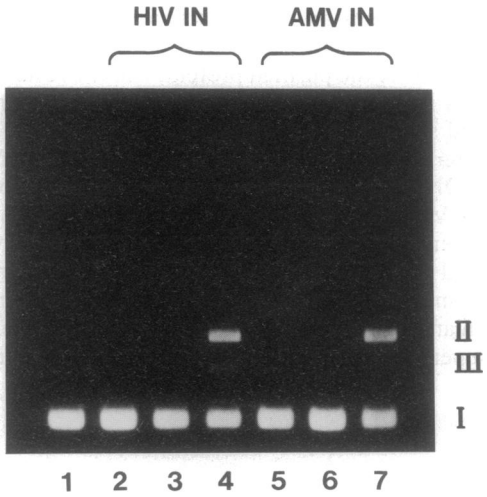

FIG. 4. Comparison of the endonucleolytic activities of HIV and AMV INs with a heterologous plasmid DNA substrate. Reaction mixtures contained the same amounts (0.2 μg) of protein. Lane 1, no protein; lanes 2–4, HIV IN; lanes 5–7, AMV IN; lanes 2 and 5, no added metal; lanes 3 and 6, 2 mM $MgCl_2$; lanes 4 and 7, 2 mM $MnCl_2$. I, supercoiled; II, nicked; III, linear.

Under these conditions, the two proteins exhibited similar specific activities; that is, equivalent amounts of the two proteins resulted in roughly equivalent product formation. The avian protein was more active than the HIV protein in the presence of Mg^{2+}. However, with both proteins the preference for Mn^{2+} over Mg^{2+} as the metal cofactor was a distinguishing feature, because this property was not observed for endonucleases of host bacterial origin.

Katzman *et al.* (18) have reported that avian IN selectively nicks double-stranded DNA oligonucleotides corresponding to the termini of linear viral DNA at sites that ultimately form the junction with the host cell DNA. This trimming reaction is presumably necessary to prepare the viral DNA for insertion into the target DNA. We, therefore, examined the ability of purified HIV IN to nick double-stranded oligonucleotides with sequences that mimic the termini of HIV and ASLV DNA. As shown in Fig. 5, HIV IN selectively cleaved the HIV U5 oligonucleotide at the 3′ end of the plus strand and the HIV U3 oligonucleotide at the 3′ end of the minus strand, at a position immediately adjacent to the conserved CA dinucleotide. The cleavage products migrated more slowly than the corresponding fragments from chemical sequencing reactions, which are known to terminate in 3′-PO_4 (19). This suggests that HIV IN produced fragments with 3′-OH termini, as was previously demonstrated in murine *in vitro* retroviral integration systems (20–22). No significant cleavage was observed on the minus strand of the HIV U5 substrate, even though it contained a fortuitous CA dinucleotide located two bases from the 3′ end. The oligonucleotide mimic of the ASLV U3 terminus was likewise not a substrate for HIV IN, although this same oligonucleotide was shown to be a substrate for avian IN (18). A substantially reduced level of cleavage was observed with an HIV U5 substrate in which the C in the conserved CA dinucleotide was replaced with a T. Surprisingly, a similar change in the DNA sequence of a murine leukemia virus resulted in only a slight reduction in the level of the 3′-recessed viral DNA precursor in infected cells and in the rate of replication compared with the wild-type virus (22).

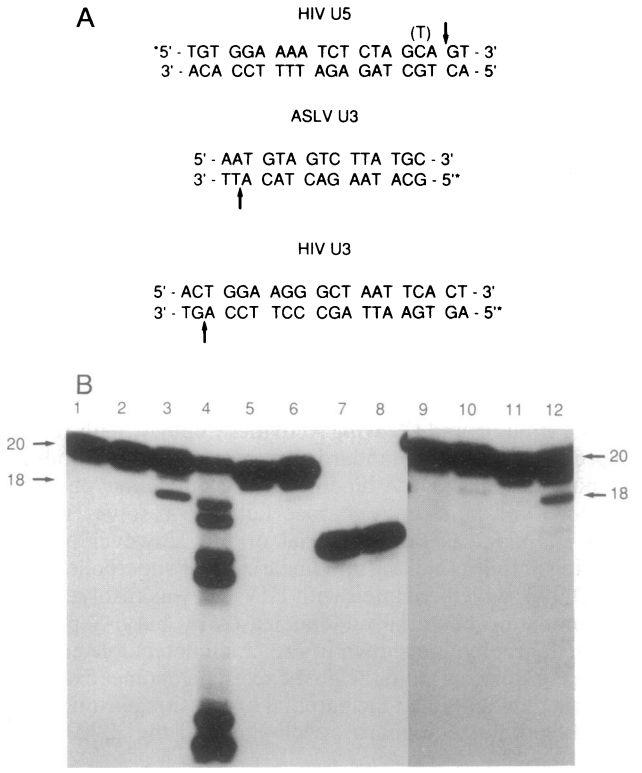

FIG. 5. Selective oligonucleotide cleaving activity of HIV IN. (*A*) Sequences of double-stranded synthetic oligonucleotide substrates. Expected cleavage sites (adjacent to the conserved CA dinucleotide) are indicated by arrows; labeled strands are indicated by asterisks. The plus strand is the upper strand of each duplex. The C to T change in the conserved CA dinucleotide is indicated above the sequence for the HIV U5 substrate. (*B*) Analysis of reaction products on denaturing polyacrylamide gels. Lanes 1–3, HIV U5 substrate with the plus strand end-labeled; lane 4, G+A Maxam–Gilbert sequencing reaction on HIV U5 substrate with the plus strand end-labeled; lanes 5 and 6, HIV U5 substrate with the minus strand end-labeled; lanes 7 and 8, ASLV U3 substrate with the minus strand end-labeled; lanes 9 and 10, HIV U5 substrate with the conserved C changed to T (plus strand end-labeled); lanes 11 and 12, HIV U3 substrate with the minus strand end-labeled; lanes 1, 5, 7, 9, and 11, no protein; lane 2, 1 mM $MgCl_2$; lanes 3, 6, 8, 10, and 12, 1 mM $MgCl_2$ plus 1 mM $MnCl_2$.

DISCUSSION

Retroviral integration, a nonhomologous recombination reaction, is thought to involve the following events. (*i*) Two bases are removed from the 3′ end of each strand of the blunt-ended linear duplex viral DNA, exposing 3′-OH groups. Such a 3′-recessed linear DNA precursor has been observed by several groups working with murine leukemia virus (20–22). (*ii*) The host cell DNA is cleaved in a staggered fashion. This staggered cut results in the short duplications of cellular DNA sequences that flank the provirus. (*iii*) The 3′-OH ends of the viral DNA are covalently joined to the 5′-PO_4 ends of the host cell DNA. (*iv*) Repair synthesis and ligation results in the mature provirus.

The role of the retroviral IN in this process has not been fully elucidated. Genetic evidence suggests that IN is required for integration (1–3). Furthermore, in cells infected with murine leukemia viruses, functional IN is required for the formation of the 3′-recessed linear DNA molecule that is the probable immediate precursor to the integrated form of the DNA (21, 22). A minimal role for IN would therefore be the selective removal of bases from the termini of the viral DNA in preparation for joining to the host cell DNA. Since most viral DNA molecules have undergone this trimming reaction before entering the nucleus (21), the generation of the 3′-recessed ends does not appear to be coupled to the joining of these ends to the target DNA. This conclusion is further supported by the observation that a synthetic mini retroviral DNA that already contains 3′-recessed ends can

serve as a substrate in a cell-free integration system (23). One can only speculate on alternative functions (i.e., other than energy conservation) of the IN-mediated cleavage of the viral DNA. IN-mediated cleavage of heterologous supercoiled plasmid DNA molecules, as has now been observed to occur with both AMV and HIV proteins, may be equivalent to the staggered cleavage of the host cell DNA. Current data would suggest that if DNA cleaving is energetically coupled to DNA joining in retroviral integration, cleavage of the target rather than the viral DNA provides the required energy. Further studies are required to determine the exact role, if any, of retroviral INs in other steps of the integration process.

While the HIV and avian integration proteins are similar in many respects, some differences are apparent. In particular, as mentioned earlier, the HIV protein exhibits less activity with Mg^{2+} as a cofactor than the avian protein. Furthermore, the HIV protein appears to be more selective than the avian protein in Mn^{2+}-dependent cleavage of oligonucleotide substrates that mimic linear viral DNA. HIV IN does not cleave an ASLV U3 substrate and selectively cleaves HIV substrates in the presence of Mn^{2+}; avian IN nonselectively cleaves HIV substrates and, less selectively than with Mg^{2+}, cleaves avian substrates (unpublished data; ref. 18). The oligonucleotide cleaving reactions discussed in this paper all contained 1 mM $MgCl_2$, which was carried over from the end-labeling reaction (see *Materials and Methods*). In one series of experiments (data not shown), Mg^{2+} was removed from the substrate by dialysis against water containing Chelex 100. Under these conditions, HIV IN did not detectably cleave the HIV U5 substrate in the absence of added metal, cleaved at a low level with added Mg^{2+}, and cleaved at a much higher level with added Mn^{2+}. The result obtained with Mn^{2+} by itself was indistinguishable from the result obtained with Mn^{2+} plus Mg^{2+}. Additional data are required to better define the selectivity differences between the HIV and avian proteins.

In summary, a putative HIV integration protein was produced in *E. coli* and purified to near homogeneity. After all detectable endonucleases originating from the host bacterial genome were removed, the HIV protein retained DNA binding and DNA cleaving activities. Further, the purified protein catalyzed one of the expected partial reactions in the integrative recombination event (that is, the removal of specific sequences at the termini of the linear viral DNA molecule in preparation for joining to the host cell DNA). A better understanding of retroviral integration proteins should aid in the development of drugs directed against the integration step in the retroviral life cycle.

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