A DNA binding assay was developed for the human immunodeficiency virus type 1 (HIV-1) integrase. This assay was capable of defining discrete complexes between the enzyme and the viral long terminal repeat (LTR) substrate. DNA binding reflected the sequence requirements previously demonstrated for the enzyme's 3'-end processing activity. Binding exhibited a nonlinear dependence on integrase concentration, suggesting that the enzyme functions as a multimer. The oligomeric state was investigated by UV-photo-cross-linking of the HIV-1 integrase-LTR oligonucleotide complexes using DNA substrates substituted with 5-bromo-2'-deoxyxycytidine within the integrase recognition sequence. In the absence of divalent cation, integrase cross-linked to the LTR oligonucleotide as a single species whose mobility by SDS-polyacrylamide gel electrophoresis was consistent with the formation of tetramers. Using these techniques, analysis of the binding properties of integrase mutants demonstrated that the catalytic and sequence-specific DNA binding activities of the enzyme are distinct, involving residues within the conserved "DD(35)E" and zinc finger motifs, respectively.

Integration of a copy of the viral genome into host cell DNA appears to be generally required for the replication of retroviruses (see Ref. 1 for review). Integration occurs in a defined series of endonucleolytic and DNA strand transfer reactions that are mediated by the virally encoded integrase protein (2-5). The site-specific endonucleolytic activity of integrase removes the 3'-terminal dinucleotide from the LTR sequences at each end of the viral genome (5-8). Subsequently, by a strand-transfer reaction, the enzyme joins the recessed 3'-termini of the viral DNA to the 5'-ends of target DNA that are generated by integrase-mediated nonspecific cleavage of the host cell genome (4, 6, 9, 10).

In several retroviral systems, including Moloney murine leukemia virus, avian sarcoma leukemia virus, Rous sarcoma virus (RSV), and the human immunodeficiency virus type 1 (HIV-1) (2, 4, 5, 7, 10-12), the development of in vitro enzyme assays, using oligonucleotide substrates and recombinantively derived integrase, has increased understanding of the aforementioned catalytic activities of integrase. The functions of specific and nonspecific endonucleolytic processing as well as DNA strand-transfer have been studied using appropriate oligonucleotide substrates and analyzing the reaction products by gel electrophoresis. The relative efficiency with which various oligonucleotide sequences serve as substrates in these reactions has been characterized in detail (11, 13-16).

Several investigators have also exploited these in vitro enzymatic reactions to study the effects of mutations in integrase in regions of the protein that are highly conserved between retroviruses (17-26). Two motifs which are of particular interest include a putative amino-terminal zinc finger and the so-called DD(35)E motif in the enzyme's "central core." The zinc finger is distinguished by 2 His and 2 Cys amino acid residues at positions 12, 16, 40, and 43, respectively (21, 27). The DD(35)E motif includes conserved Asp residues at positions 64 and 121 and a conserved Glu residue at 152 (17, 21, 26, 28). Lesions within the zinc finger decrease the specific endonucleolytic and integration activities of the HIV-1 enzyme, but have limited effect on nonspecific DNA cleavage (17, 18, 26). In contrast, mutations in the DD(35)E motif eliminate all in vitro enzymatic activity (17, 18, 24-26). Although these data suggest that the specific endonucleolytic and DNA strand-transfer activities of integrase involve a common active site, the trivial explanation that these mutations merely affect the protein's structural integrity cannot be discounted. In addition, previous studies did not address whether the substitutions affect substrate binding and/or catalytic function. An additional factor complicating the interpretation of these studies derives from biophysical evidence that integrase is oligomeric (5, 18, 29). Depending on the source of enzyme and method of analysis, both dimeric and tetrameric forms of the enzyme have been observed (5, 18, 29). Although the precise relationship between oligomeric structure and function is unknown, biochemical studies using the RSV enzyme suggest that at least a dimer of integrase is required for both the processing and DNA strand-transfer reactions (29).

Accordingly, a DNA binding assay for the HIV-1 integrase was developed to address questions regarding DNA substrate recognition and subunit composition. Unlike previously published binding assays (12, 21), this assay was able to discern a discrete, enzymatically relevant complex between integrase and the HIV-1 LTR substrate. The nature of the oligomeric state of integrase in these complexes in relationship to substrate binding and divalent cation composition was investigated by UV-photo-cross-linking. Finally, these techniques are used to analyze integrase mutants containing substitutions in either the zinc finger or DD(35)E domains, suggesting that the former participates in specific substrate recognition, while the DD(35)E motif is essential for enzymatic activity.

MATERIALS AND METHODS

Cloning, Expression, and Purification of Wild-type and Mutant Integrases—Cloning of the wild-type HIV-1 integrase protein and expression by a T7 expression vector were described previously (11). Mutation of the integrase and characterization of the cleavage and strand-transfer properties of the mutant enzymes were also described (17). Both wild-

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‡ The abbreviations used are: LTR, long terminal repeat; RSV, Rous sarcoma virus; Brd(dC), 5-bromo-2'-deoxyxycytidine; PAGE, polyacrylamide gel electrophoresis.
Binding Assay—A 20-base pair oligonucleotide (Midland Scientific, Midland, TX) was labeled at the 5' end by [y-32P]ATP (5000 Ci/mmol, Amersham Corp.) using T4 polynucleotide kinase (Pharmacia LKB Bio-technology Inc.) as described (17). Binding reactions between the oligonucleotide and mutant proteins were purified by the protocol of Sherman and Fye (5).

The position of the photoactive Br(dC) substitution is noted in the U3 HXB2 LTR oligonucleotide substrate (Fig. 2), representing the terminus of the HIV-1 HxB2 U3 LTR, was labeled at the 5' end by [y-32P]ATP (>5000 Ci/mmol, Amersham Corp.) using T4 polynucleotide kinase (Pharmacia LKB Biotechnology Inc.) as described (17). Binding reactions between the oligonucleotide and the purified integrase were performed in the absence of Mg2+ or Mn2+ in binding buffer (20 mM Tris HCl, pH 7.9, 0.1 M NaCl, 0.05 mg/mL bovine serum albumin, 5.0 mM 2-mercaptoethanol). Labeled oligonucleotides were added at a concentration of 1.0 nM. Unlabeled competitor oligonucleotides were added to the reactions prior to integrase at the concentrations noted in the figure legends. Binding reactions were initiated by the addition of wild-type or mutant integrase, again as noted in the figure legends. Following 30 min at 4°C for 2 h, the complexes were visualized by autoradiography. Protein band quantification was performed using the AMBIS Radioanalytic Imaging System (AMBIS, San Diego, CA).

For experiments in which antisera was used, 1 μl of the appropriate antisera was added subsequent to the 30-min binding reaction. The reactions were incubated for an additional 30 min on ice and then analyzed on nondenaturing gels as described above. Rabbit polyclonal antisera to HIV-1 integrase residues 1–16, 23–34, and 276–288 were obtained from D. P. Grandgenett through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. These antibodies are both Western blot and immunoprecipitation-reactive. Rabbit antisera to HIV-1 integrase were also prepared against synthetic peptides encompassing residues 6–23 and 270–288. These antisera were shown to specifically recognize a 33-kDa protein in Western blot analysis of lysates from recombinant Escherichia coli expressing either wild-type or mutant integrase. No immunoreactive proteins were detected using control lysates or preimmune sera (not shown).

**Fig. 1. Silver stain and Western analysis of purified wild-type and mutant integrase proteins.** For each protein, wild-type (WT) or mutant as indicated, 250 ng of total protein was loaded per lane and electrophoresed on 12.5% SDS-PAGE. A, silver stain analysis of proteins. B, Western analysis using antisera to peptide (270–288) described under "Materials and Methods."
cations. Binding reactions were performed using wild-type integrase. The concentration of integrase in each reaction was varied according to the conditions used for the DNA binding reaction. Quantification of the discrete complex (CI) and the incorporation of radiolabeled U3 LTR sequences were performed as outlined in Fig. 3 except that after the 30 min on ice, 1 μl of antisera was added as follows: A: lane 1, no antisera; lane 2, α1-16; lane 3, α20-34; lane 4, α276-288; lane 5, α6-23; lane 6, α270-288; lane 7, α270-288. B: lane 1, preimmune (6-23); lane 2, preimmune (270-288); lane 3, α6-23; lane 4, α270-288.

Substrate Recognition Parallels Substrate Utilization—To determine if the discrete integrase-substrate complex formed in the absence of Mg²⁺ or Mn²⁺ actually reflected relevant substrate recognition of viral DNA sequences by the enzyme, competition studies were performed using both an oligonucleotide representing wild-type U5 LTR sequence and a mutant U5 viral LTR oligonucleotide (Fig. 2) containing substitutions that significantly reduce its ability to be processed by integrase (11). The ability of these oligonucleotides to compete with the radiolabeled U3 LTR substrate for integrase binding was assessed in the gel mobility shift assay. The results of these studies showed that the wild-type U5 LTR competed effectively with the U3 LTR for binding, while the mutant U5 sequence was significantly reduced (approximately 9-fold) in its ability to compete for binding to the wild type sequence (Fig. 4). It had been shown previously that the U5 LTR is indistinguishable from the U3 LTR as a substrate for integrase-mediated cleavage, while the mutant LTR sequence is cleaved 20-fold less efficiently (11). Therefore, in the gel mobility shift assay, the ability of the integrase to bind a specific substrate DNA sequence and form a discrete complex appears to correlate with the sequence-specific recognition required for enzymatic function.

Integrase Tetramers Bind to the LTR—Analysis of the relationship between integrase concentration and integrase-LTR substrate complex formation (Fig. 3B) suggested that the binding of the enzyme to the substrate is nonlinearly dependent on integrase concentration. Therefore, binding to the LTR may require a multimeric form of the enzyme. Both the endonucleolytic and DNA strand-transfer activities of the RSV integrase also display a distinct nonlinear dependence on enzyme concentration (29). Sedimentation studies have shown that the RSV enzyme exists in equilibrium among monomeric, dimeric, and tetrameric forms, with a Kₐ for multimerization of about 1.0 μM (29). If the HIV-1 integrase is similar to the RSV protein in this regard, several multimeric forms of the HIV-1 enzyme should be present in the DNA binding reactions at the enzyme concentrations used. However, since only a single complex between the integrase and the LTR oligonucleotide was detected in the gel mobility shift assay (Figs. 3, 4, and 5), only one form of the HIV-1 enzyme may be competent to bind the viral DNA.

To determine which multimeric form of the integrase is bound to the LTR substrate, photo-cross-linking studies were performed using a U3 LTR oligonucleotide that included a UV photoactivatable group (Br(dC)) within the integrase recognition bindable substrate due to processing by the enzyme. Minimal catalysis occurs under the conditions used for the DNA binding reaction (i.e. at 4 °C), and analysis of the substrate isolated from the discrete complex assembled in the absence of divalent cation or the undefined complexes formed in the presence of Mn²⁺ confirmed that the integrase-associated LTR sequences were neither processed nor integrated (data not shown). Moreover, integrase binds to LTR oligonucleotides with 3'-recessed termini with an efficiency similar to unprocessed substrates (data not shown).

Although the preparation of recombinant integrase used in these studies was greater than 90% pure (Fig. 1), it was possible that the observed retarded complex was the result of an interaction between the LTR oligonucleotide and a low abundance but high affinity contaminant protein. To address this concern, antibodies elicited by peptides representing either the amino or carboxyl terminus of integrase were added to the binding reaction prior to analysis. As shown in Fig. 4, these antibodies both reduced the amount of the complex and further decreased its electrophoretic mobility. In contrast, preimmune sera had no effect (Fig. 4B, compare lanes 1 and 2 with lanes 3 and 4). These data demonstrate that the complex detected in the gel mobility shift assay resulted specifically from the interaction of integrase with the LTR oligonucleotide and was not due to a contaminating protein.
Substrate Binding by HIV-1 Integrase

Figure 5. Competition of U3 LTR oligonucleotide binding to integrase by wild-type (WT) or mutant (CGT) U5 LTR oligonucleotides. Nondenaturing gel electrophoresis of the DNA binding reactions are shown. Reactions were performed using radiolabeled U3 LTR oligonucleotide under standard conditions (see text) with the addition of unlabeled competitor oligonucleotides (shown in Fig. 1). The concentration of competitor DNA varied from 1.6 to 25 ng as indicated. Integrase was used at a concentration of 100 ng.

Figure 6. UV-photo-cross-linking of HIV-1 integrase to Br(dC)-substituted substrate oligonucleotide. The cross-linking reaction was performed in the presence or absence of 3 mM MnCl₂ as indicated. The reaction products (CI, CII, and CIII) were analyzed as described under "Materials and Methods." The concentrations of integrase in the reactions were 0, 50, and 60 ng (lanes 1–3 and 4–6, respectively). B shows the quantification and molecular weight determination from the results in A.

The LTR Binding and Catalytic Activities of Integrase Are Distinct—Having developed assays competent to assess both the DNA binding and multimerization properties of integrase, the functional lesions of integrase mutants previously shown to be deficient in catalytic activity can be analyzed. Mutations within the highly conserved regions of integrase, the putative zinc finger and the "DD(35)E" core, are of particular interest. Mutations in either of these conserved motifs adversely affect the specific endonuclease and/or integrative properties of the enzyme (16, 17, 23–25). However, while mutations in the DD(35)E motif affect both the "specific" and "nonspecific" catalytic activities of integrase, mutations in the zinc finger appear to influence only those activities of integrase which involve viral DNA sequences, i.e. cleavage of the LTR and integration.

We used the gel retardation and cross-linking assays to investigate the DNA binding phenotype of two integrase mutants, C43S and V151E,D152Q whose catalytic properties were described previously (11). The former mutant involves a conserved residue within the zinc finger, while the latter is a double substitution of highly conserved amino acids in the central core. As detailed elsewhere, these mutant enzymes were expressed and purified equivalently to the wild-type protein, suggesting that the mutations did not result in a gross structural alteration of the enzyme (11). All proteins were purified to greater than 90% homogeneity and were used at identical concentrations (Fig. 1).

Examination of the LTR DNA binding activity of the V151E,D152Q mutant by gel retardation demonstrated that the binding and catalytic functions of the integrase are separable. Although the mutant protein is enzymatically nonfunctional, it exhibited wild-type LTR substrate binding activity (Fig. 7). Moreover, since the electrophoretic mobility of the mutant integrase complex was indistinguishable from the wild type integrase complex, the V151E,D152Q mutant and wild-type enzyme complexes assume the same multimeric state. As expected, UV-photo-cross-linking of the mutant protein to the Br(dC)-substituted LTR oligonucleotide showed that, like the wild-type enzyme, the mutant integrase forms a tetrameric complex with the oligonucleotide substrate (Fig. 8). Therefore, the inability of the mutant to express catalytic activity is not due to a defect in either appropriate substrate binding or multimer assembly.

The Zinc Finger of Integrase Mediates Specific Interaction with the Viral LTR Substrate—In contrast to the V151E,D152Q enzyme which is enzymatically inert, an integrase mutant containing a substitution (Cys → Ser) within the enzyme's zinc finger domain at residue position 43, described by LaFemina et al. (17), exhibits normal levels of nonspecific endonucleolytic activity, but its ability to mediate specific removal of the terminal LTR dinucleotide is reduced by 90% (17). This mutant enzyme also exhibits significantly reduced DNA strand-transfer activity, at least in part due to the inability to specifically process the integration substrate. As demonstrated both by the DNA binding assay shown in Fig. 7 and by the UV-photo-cross-linking experiment shown in Fig. 8, this mutant enzyme dis-
DISCUSSION

Two novel assay systems were developed to study the interaction between the HIV-1 integrase and its viral LTR substrate. The assays, performed in the absence of divalent cations using purified enzyme and oligonucleotides representing the U3 and U5 LTR ends, showed that integrase binds the LTR substrate forming a discrete complex in which the enzyme exists as a tetramer. Formation of the complex correlated with sequence-specific recognition of the viral LTR substrate and specific endonucleolytic and DNA strand-transfer activities.

This is the first report to demonstrate defined complexes between the HIV-1 integrase and viral LTR oligonucleotide substrates. Previous attempts to study LTR binding were typically performed under conditions favoring enzyme catalysis (12, 21). As a result, the stable formation of complexes may have been limited by normal enzymatic turnover. The assay described here minimized turnover by eliminating the divalent cations required for enzyme activity. The only other report of sequence-specific DNA binding by a retroviral integrase was published by Krogstad and Champoux (31) using the Moloney murine leukemia virus enzyme. These experiments were also performed under experimental conditions not favorable to enzymatic function.

In addition to limiting the enzymatic activity during binding, the concentration of labeled LTR substrate oligonucleotide used in the present studies was also lowered significantly (approximately 10-fold) relative to the concentrations reported for previously described assays (11, 12). Kinetic analyses of DNA binding suggest that the $K_m$ for the specific LTR substrate is 5.0 nM, while the $K_m$ for nonspecific DNA substrates is 6- to 10-fold higher. Therefore, as the concentration of the LTR substrate oligonucleotide approaches the $K_m$ for nonspecific binding, the difference between the two modes of enzyme-substrate interaction is diminished. Since most published assays rely on the LTR substrate for both the specific and nonspecific endonucleolytic events required for strand transfer, conditions have been optimized such that the concentration of the LTR oligonucleotide is not limiting as a nonspecific substrate for integration. In contrast, the lower concentration of the substrate used in the present assay should favor the specific endonucleolytic event. Lower LTR substrate concentrations do in fact promote the generation of the specific cleavage product while, at higher concentrations, a significant proportion of the cleavage products are nonspecific.

We have demonstrated that, under specific conditions, integrase forms a discrete tetrameric complex with the LTR substrate oligonucleotide. Previous biophysical studies have shown that retroviral integrases exist in solution as monomers and dimers, as well as tetramers (5, 18, 29). The DNA binding and photo-cross-linking experiments reported here demonstrated that in the absence of divalent cation, a single oligomeric species of integrase interacts with LTR substrate oligonucleotide, suggesting that this species, the tetrameric form of the enzyme, is functional. However, this observation is limited by the substrate used and may apply only to the enzyme's specific endonucleolytic activity. It may not extend to DNA strand-transfer which requires integrase interaction with more than one DNA molecule.

The assembly of the DNA strand-transfer complex would likely be mediated through protein-protein interaction between integrase oligomers bound to the respective donor and target DNA substrates. Since the largest multimer of the integrase identified through biophysical methods is the tetramer, it has been suggested that this form is also probably involved in the strand-transfer reaction (29). It should be noted, however, that both the sedimentation and gel filtration studies previously published were performed in high salt and in the absence of divalent cation (18, 29). Since high salt inhibits enzymatic activity (18) and either Mn$^{2+}$ or Mg$^{2+}$ are absolutely required for catalysis, it is possible that these higher order interactions can only be observed under appropriate buffer conditions. Alternatively, higher order interactions may require assembly on the appropriate substrate(s). Our observation that the addition of Mn$^{2+}$ or Mg$^{2+}$ promotes the formation of large nucleoprotein complexes composed of monomeric, dimeric, and/or tetrameric forms of integrase bound to DNA suggests that the integration complex may require interactions beyond the tetramer. Whether these interactions require prior binding to the appropriate substrates is currently under investigation.

We used the DNA binding assay to show that a mutant form of the HIV-1 integrase, containing a substitution within the enzyme's DD(35)D motif, was indistinguishable from the wild-type protein in its ability to form a specific complex with the LTR substrate. Nonetheless, the mutant enzyme is deficient for all catalytic activity (17) as are other enzymes with amino acid substitutions in this motif (24). Hence, the DD(35)E motif, which is highly conserved among retroviral integrases, is essential for integrase catalytic function, but does not participate in substrate recognition.

1 D. J. Hazuda, A. L. Wolfe, J. C. Hastings, H. L. Robbins, P. L. Graham, R. L. LaFemina, and E. A. Emini, unpublished observations.

2 D. J. Hazuda, A. L. Wolfe, J. C. Hastings, H. L. Robbins, P. L. Graham, R. L. LaFemina, and E. A. Emini, unpublished observations.
Finally, the DNA binding assay was also used to demonstrate that a HIV-1 integrase mutant containing a substitution within the enzyme's zinc finger is deficient in its ability to form complex with the LTR substrate. This deficiency was consistent with the mutant's inability to catalyze the specific endonuclease reaction (17). However, the non-specific endonucleolytic activity of this enzyme is not impaired (17), as is the non-specific DNA binding of such zinc finger mutants (21). Therefore, the zinc finger of integrase is functionally analogous to the zinc finger of the TFIIA transcription factor, in which zinc is essential for the specific interaction of the factor with the 5 S RNA gene but not for non-specific interactions with DNA (32).

Most sequence-specific DNA binding proteins in which zinc fingers have been identified function as multimers and/or have multiple zinc fingers (33). Studies involving deletion or mutation of individual zinc fingers in multiple-finger proteins have shown that more than one finger is needed to maintain the requisite number of correct base contacts (33). Therefore, the specificity and affinity of protein-DNA binding is probably attained through the cooperative effect of multiple zinc finger interactions. Given that the HIV-1 integrase monomer has only one zinc finger, multimerization of the enzyme may be essential for sequence-specific DNA binding.

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