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Isolation and Characterization of an Oligomerization-Negative Mutant of HIV-1 Integrase

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The yeast two-hybrid method was used to screen mutagenized DNAs to isolate a variant of the human immunodeficiency virus type 1 integrase (IN) that does not interact with the wild-type IN. The responsible mutation, leading to a single amino acid change (V260E) in the C-terminal domain of IN, blocks IN–IN multimerization but has only small effect on binding to a host interacting protein, INI1 (hSNF5). Binding studies *in vitro* confirmed the defect in multimerization of the mutant IN. Biochemical analyses of the mutant IN enzyme expressed in bacteria detected only subtle changes in its properties, suggesting that the yeast system is a sensitive reporter of correct IN conformation. Mutant virus carrying the V260E substitution was blocked in replication at the time of DNA integration, consistent with IN multimerization being important for its activity *in vivo.*

Integration of a DNA copy of the viral genome into the host chromosome is an essential step in the life cycle of retroviruses (for reviews see Andrake and Skalka, 1996; Asante-Appiah and Skalka, 1997; Goff, 1992; Vink and Plasterk, 1994). After retrovirus particles enter a host cell, the RNA genome is reverse-transcribed to form a linear double-stranded DNA that is subsequently integrated into the host genome to form the provirus. A single viral enzyme, integrase (IN), mediates two major steps in the process of integration (Katz *et al.,* 1990). In the first step, two bases from the 3′ ends of each DNA terminus are removed, leaving exposed 3′ hydroxyl groups (3′ processing); in the second step, the 3′ ends of the viral DNA are joined to 5′ phosphate groups of the host DNA (strand transfer; Brown *et al.,* 1989; Bushman and Craigie, 1991; Engelman *et al.,* 1991). Most *in vitro* reactions using recombinant IN are typically half-reactions that demonstrate correct cutting and processing of only one LTR end (Bushman *et al.,* 1990; Craigie *et al.,* 1990; Fujiwara and Craigie, 1989; Fujiwara and Mizuuchi, 1988; Katz *et al.,* 1990; Katzman *et al.,* 1989). In an infected cell the two ends must be inserted into the target in a concerted reaction, and multiple lines of evidence suggest that integrase functions as an oligomer to act on both DNA ends. In solution, IN exists as a mixture of monomers, dimers, and tetramers in equilibrium with the relative amounts dependent on the concentration (Jenkins *et al.,* 1996; van Gent *et al.,* 1991). Direct examination of recombinant RSV integrase coupled with kinetic studies indicates that the active form is an oligomer (Jones *et al.,* 1992); this oligomerization is stimulated by Zn^{2+} (Lee *et al.,* 1997; Zheng *et al.,* 1996) and Mn^{2+} (Wolfe *et al.,* 1996). Complementation experiments with mixtures of defective and wild-type HIV-1 integrase also indicate that a multimeric form of the enzyme catalyzes the integrase reaction *in vitro* (Engelman *et al.,* 1993; van Gent *et al.,* 1993), and protein cross-linking has further shown that IN exists as a multimer (Engelman *et al.,* 1993; Heuer and Brown, 1997, 1998). Finally, tests of protein–protein interactions in the yeast two-hybrid system have directly demonstrated that integrase can oligomerize (Kalpana and Goff, 1993).

Comparisons of the integrase sequences of various retroviruses and retrotransposons as well as biochemical studies indicate that integrase has three distinct domains (Bushman *et al.,* 1993; Engelman *et al.,* 1993; Engelman and Craigie, 1992; Khan *et al.,* 1991; Leavitt *et al.,* 1993; Vincent *et al.,* 1993; Vink *et al.,* 1993): an N-terminal zinc finger-like domain (typically contained in aa 1–50), a central core domain with a conserved D,D35E motif (aa 50–200), and a poorly conserved C-terminal domain with nonspecific DNA binding activity. Probably all three domains of IN are involved in some aspect of IN–IN multimerization (Andrake and Skalka, 1995; Barsov *et al.,* 1996; Ellison *et al.,* 1995; Jenkins *et al.,* 1996). Direct structure analysis suggests that the N-terminal region is a dimer in solution (Cai *et al.,* 1997). Biochemical complementation studies suggest that the central core domain is involved in multimerization (Engelman *et al.,* 1993; van Gent *et al.,* 1993). In addition, analyses of pools of detected integrase clones in the yeast two-hybrid system indicate that the central core domain is essential for

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oligomerization, though there are some requirements for adjacent domains (Kalpana and Goff, 1993). The results of these biochemical and genetic studies are supported by the X-ray crystal structure of the integrase core domain (Dyda *et al.,* 1994). In this crystal structure, the central core domain of integrase formed a dimer with extensive interactions throughout the length of the core polypeptide. The C-terminal domain of the avian viral IN has been demonstrated to self-associate by size-exclusion chromatography, chemical cross-linking, and protein blot (Andrake and Skalka, 1995). NMR studies have confirmed that the C-terminal domain is a dimer in solution; the domain is composed of a five-stranded β-barrel with topology similar to that of the SH3 domain (Eijkelenboom *et al.,* 1995; Lodi *et al.,* 1995).

The overall oligomeric state of IN *in vivo* and the specific residues required for oligomeric contacts at the IN–IN interfaces remain uncertain. We have now used the yeast two-hybrid system (Chien *et al.,* 1991; Fields and Song, 1989) as a screening assay to identify specific residues involved in multimeric interactions of IN. In this system, a library of IN mutants fused to the Gal4 DNA binding domain (GAL4DB) is coexpressed with wild-type IN fused to the Gal4 activation domain (GAL4AC) in an indicator yeast strain. As reported previously, wild-type IN multimerizes in this system, resulting in the formation of blue colonies in the X-gal assay (Kalpana and Goff, 1993). Those rare IN mutants unable to interact with wild-type IN result in white colonies in the X-gal assay. Using this screening assay, we have identified a point mutation that causes an interaction-negative phenotype. The mutant IN binds to wild-type IN poorly *in vitro,* exhibits slightly decreased 3′ processing and joining activities, and exhibits disintegration activity comparable to that of wild-type IN *in vitro,* but does not support viral integration in the context of the viral genome. The results suggest that the multimeric interacting structures formed in the yeast system are important for normal IN function *in vivo.*

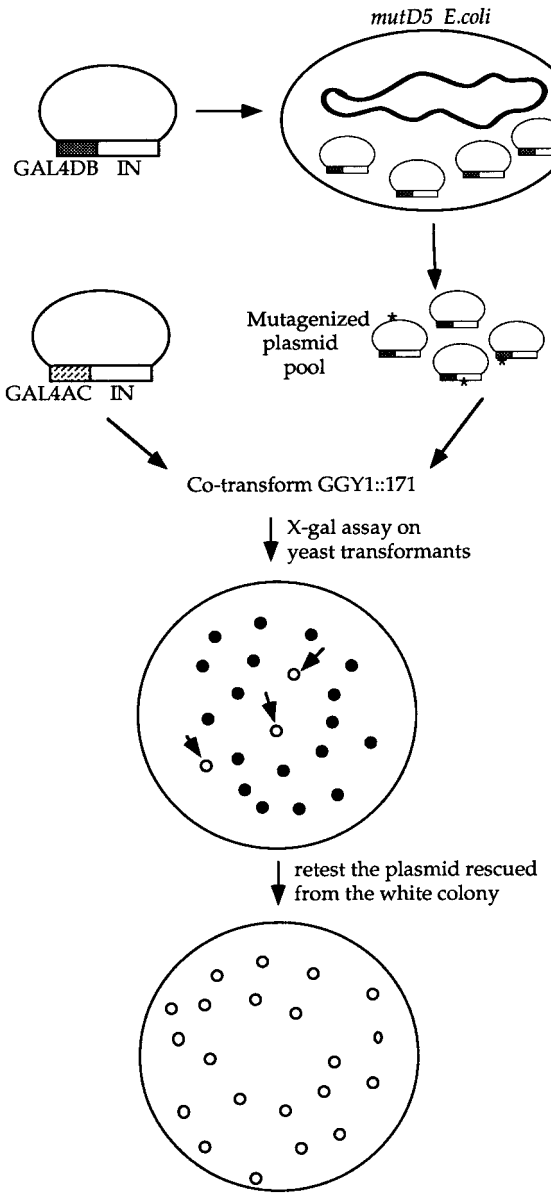

FIG. 1. Strategy for the isolation of interaction-negative mutants of IN. GAL4DB–IN expression plasmid (top) was mutagenized by passage through a mutator *E. coli.* The library of mutant plasmids, along with unmutagenized GAL4AC–IN, was introduced into the yeast reporter strain GGY1::171, and the resulting yeast transformants were stained with X-gal. Plasmid DNAs isolated from the white colonies (indicated by the arrow) were retested and subjected to further analysis.

RESULTS

Isolation of interaction-negative mutants of IN

We have previously demonstrated that the multimerization of HIV-1 IN can be monitored by using the yeast two-hybrid system: the coexpression of GAL4DB-IN along with GAL4AC-IN in yeast results in the formation of functional activation complex that can be detected by the transactivation of a reporter *lacZ* gene (Kalpana and Goff, 1993). To isolate interaction-negative mutants of IN, a yeast plasmid encoding GAL4DB-IN (pMAI, Kalpana and Goff, 1993) was subjected to mutagenesis by passage in a mutator strain of *Escherichia coli* (see Materials and Methods). The pool of mutagenized DNA was then used to transform the GGY1::171 yeast reporter strain along with an unmutagenized plasmid encoding wild-type GAL4AC-IN (pGADI; Fig. 1). Colonies were lifted to nitrocellulose and stained with X-gal. Of 2000 colonies screened, 21 white colonies containing candidate mutants were detected. To confirm the interaction-negative phenotype, plasmids were isolated from these 21 colonies, recovered by cloning in *E. coli,* and retested by transformation of the GGY1::171 strain with GAL4AC-IN DNA. Fourteen of the clones resulted in Lac^- colonies, while the remaining 7 gave Lac^+ colonies and were discarded. Restriction analysis of the 14 clones indicated that 7 had undergone spontaneous deletion of the GAL4

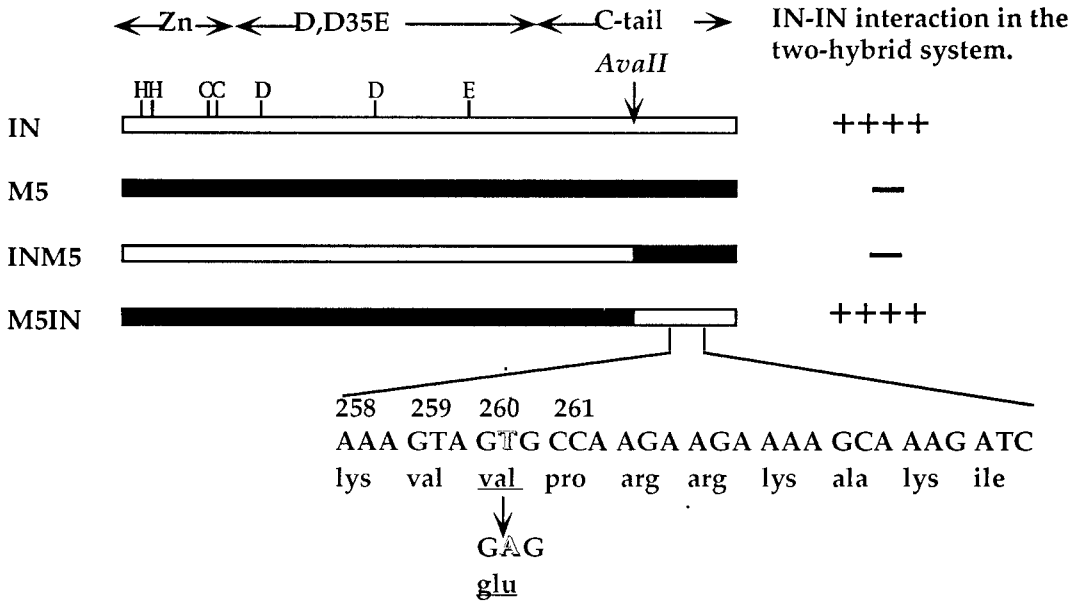

FIG. 2. Position of interaction-negative mutation in IN. The conserved domains of IN are indicated at the top. The white bar indicates the wild-type IN; filled bar represents the mutant M5; the INM5 and M5IN bars represent chimeras containing IN and M5 fragments. A blow-up of the C-terminus is shown at the bottom. The numbers indicate the IN residues. The change in the sequence is indicated by an arrow and shaded letters. Zn, zinc finger domain; D,D35E, the central domain with catalytic residues; C-tail, C-terminal domain. Amino acid residues indicated are H, histidine; C, cysteine; D, aspartic acid; E, glutamic acid. The IN mutants were tested in the two-hybrid system as GAL4DB fusion proteins against GAL4AC–IN fusion protein. A plus sign indicates blue colonies, and a minus sign indicates white colonies in the X-Gal assay.

region. DNA fragments containing the IN region from the remaining 7 clones were isolated, recloned into the GAL4DB plasmid, and retested for their ability to transactivate *lacZ* when cotransformed with the GAL4AC-IN plasmid. Four of the 7 clones resulted in blue colonies, indicating that the original mutations in these clones resided elsewhere in the plasmid and not in the IN region. To determine whether the remaining 3 clones could direct the synthesis of full-length protein, the IN fragment was cloned into a T7 expression vector, and expression of the mutant proteins was assessed by Western analysis of lysates using IN antibodies. Only 1 of the 3 clones (M5) expressed full-length IN protein, indicating that mutations in the other 2 clones had caused truncation of the protein or affected protein stability. The mutant M5 was characterized further.

Determining the change in the M5 mutant

The entire coding regions of both M5 and the wild-type IN were subjected to sequence analysis and compared. A single A-to-T transversion mutation was found near the 3′ end of the mutant DNA; this mutation results in a V260E substitution. There was no other alteration in the sequence. To confirm that the V260E substitution was responsible for the interaction-negative phenotype, a small 3′ fragment of M5 and the wild-type clone were swapped, and the resulting chimeras were tested for oligomerization in the two-hybrid system (Fig. 2). The C-terminal fragment containing the mutation was able to confer the interaction-negative phenotype to the chimera, indicating that the substitution mutation was the cause of the observed phenotype.

Interaction properties of the V260E mutant

The M5 mutant was originally isolated as a GAL4DB fusion protein that was unable to interact with IN fused to GAL4AC. To test whether the interaction-negative phenotype was specific to the GAL4DB–IN fusion protein, and also to determine whether the mutant was able to homodimerize, the mutant IN protein was expressed as a GAL4AC fusion. All combinations of mutants and IN proteins fused to either GAL4DB or GAL4AC were coexpressed and tested for interaction (Fig. 3). The mutant M5 was unable to interact either with itself or with wild-type IN in any combination. In addition, we also tested to see whether the M5 mutant interacted with the integrase interactor 1 (INI1; hSNF5), a host protein first identified as binding to the HIV-1 IN (Kalpana *et al.,* 1994). The M5 IN did indeed interact with INI1, although the activation of *lacZ* was slightly weaker than seen with the wild-type IN–INI1 interaction (Fig. 3). This result indicates that M5 makes a stable protein that is well folded, targeted to the nucleus, and capable of interacting with some proteins, but is specifically blocked in homomeric interactions in the two-hybrid system. The results suggest that M5 is a true interaction-negative mutant.

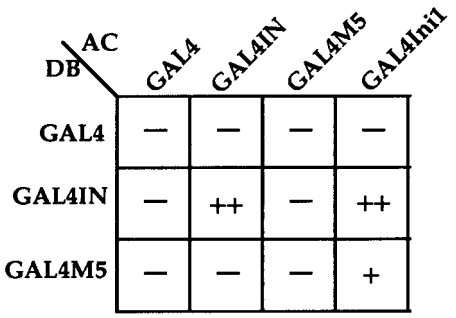

FIG. 3. Interaction of M5 mutant with IN and Ini1 in the two-hybrid system. DB, fusions to DNA binding domain; AC, fusion to activation domain. The corresponding pairs of fusion proteins were tested for the interaction in the two-hybrid system and the results of the X-gal assay are indicated. ++, dark blue, +, light blue, and −, white. IN, wild-type IN; M5, interaction-negative mutant; Ini1, fragment of Ini1 (aa 105–385) that binds to IN.

Effect of the V260E substitution on *in vitro* integration activities

Recombinant IN protein is able to catalyze three specific reactions: 3′ processing of the viral DNA termini, joining of a processed terminus to a target DNA, and disintegration, a reverse reaction in which a viral terminus is released from its target. To determine whether the V260E substitution affected 3′ processing, joining, or disintegration activities *in vitro,* the wild-type IN and M5 were expressed with a hexa-histidine (6H) tag in bacteria and affinity-purified on Ni-NTA Sepharose columns. In addition, the two IN proteins were also expressed as GST fusions and purified on glutathione beads. Processing reactions were carried out under standard assay conditions, with double-stranded labeled oligonucleotides corresponding to the U5 end of HIV-1 DNA as substrate, using similar amounts of 6H-IN and 6H-M5 (Fig. 4a). To test for DNA joining activity, a labeled preprocessed oligonucleotide corresponding to the U5 terminus was used as a substrate and an unlabeled plasmid DNA was used as target (Kalpana *et al.,* 1994). Integration of the radiolabeled substrate into the target was detected by autoradiography after gel electrophoresis as labeled, tailed, circular molecules (Fig. 4b). Normalized for the amount of protein added, the mutant exhibited about threefold less processing and joining activity compared to the wild-type, indicating that the V260E mutation modestly reduced the catalytic activity of IN. It has been demonstrated that IN can carry out disintegration reactions even when multimerization is prevented and under conditions when the 3′ processing and joining reactions are very inefficient (Barsov *et al.,* 1996; Engelman and Craigie, 1992). To test for disintegration activity, reactions were performed using a Y substrate labeled at the 5′ end (Chow *et al.,* 1992); during the reaction a 16-nt labeled oligonucleotide substrate is joined to form a 30-nt radiolabeled product. The 6H–IN and GST–IN proteins both showed high levels of activity (Fig. 4c). The GST-M5 also showed strong activity, roughly in proportion to the amount of protein used in these assays. The results indicate that the V260E mutation did not grossly affect the enzymatic activity, the catalytic site, or the overall folding of the protein and suggest that the primary defect is in multimerization.

In vitro interactions of IN and M5

To determine whether the defect in oligomerization in the two-hybrid system could be detected *in vitro,* we developed a simple binding assay using GST–IN immobilized on beads and hexa-His tagged mutant and wild-type IN in solution. Glutathione beads were loaded with GST, GST–IN, or GST–INI1 proteins and incubated with bacterial lysates containing 6H-M5 or 6H-IN proteins. The beads were washed extensively and the bound proteins were then eluted, separated by SDS gel electrophoresis, and analyzed by immunoblotting with polyclonal anti-6H antibodies. The GST–IN beads bound the wild-type 6H-IN efficiently (Fig. 5, lane 1), but bound the 6H-M5 mutant protein only very poorly (lane 2). Although the M5 lysates contained somewhat less protein than the wild-type lysates (approximately 2-fold less), quantitation showed that the beads bound 40-fold less of the M5 than the wild-type IN, suggesting a severe defect in the interaction. As a control, the binding to a distinct IN-interacting protein, INI1 (hSNF5), was examined. The GST–INI1 beads bound both the wild-type 6H-IN and the 6H-M5 proteins efficiently, indicating that these proteins were capable of interacting; in fact, the beads bound the M5 mutant protein even more efficiently than the wild-type (Fig. 5, lanes 3 and 4). The GST beads showed no nonspecific binding of either protein. These results show that the M5 mutant integrase was specifically impaired in its interaction with the wild-type IN in this *in vitro* assay, correlating with its defect in the yeast system.

Analysis of effects of the M5 mutation on viral replication

IN is essential for productive infection by HIV-1 (LaFemina *et al.,* 1992; Sakai *et al.,* 1993; Shin *et al.,* 1994; Wiskerchen and Muesing, 1995). To determine whether the M5 mutant IN protein could function *in vivo,* the M5 mutation was introduced into a molecular clone of the HIV-1 provirus. The ability of the mutant to assemble and release virions with properly processed Gag and Pol proteins was assessed after transfection of COS7 cells with wild-type or mutant proviral DNA. Sixty hours post-transfection, culture supernatants were harvested, filtered, and concentrated, and Western analysis of viral proteins was carried out using equal volumes of concentrated virions. To detect the mature cleavage products of $Pr160^{gag\text{-}pol}$ and $Pr55^{gag}$, a polyclonal antiserum from HIV-1-infected patients was used, and to detect processed IN protein, a monoclonal anti-IN antiserum was used (Figs.

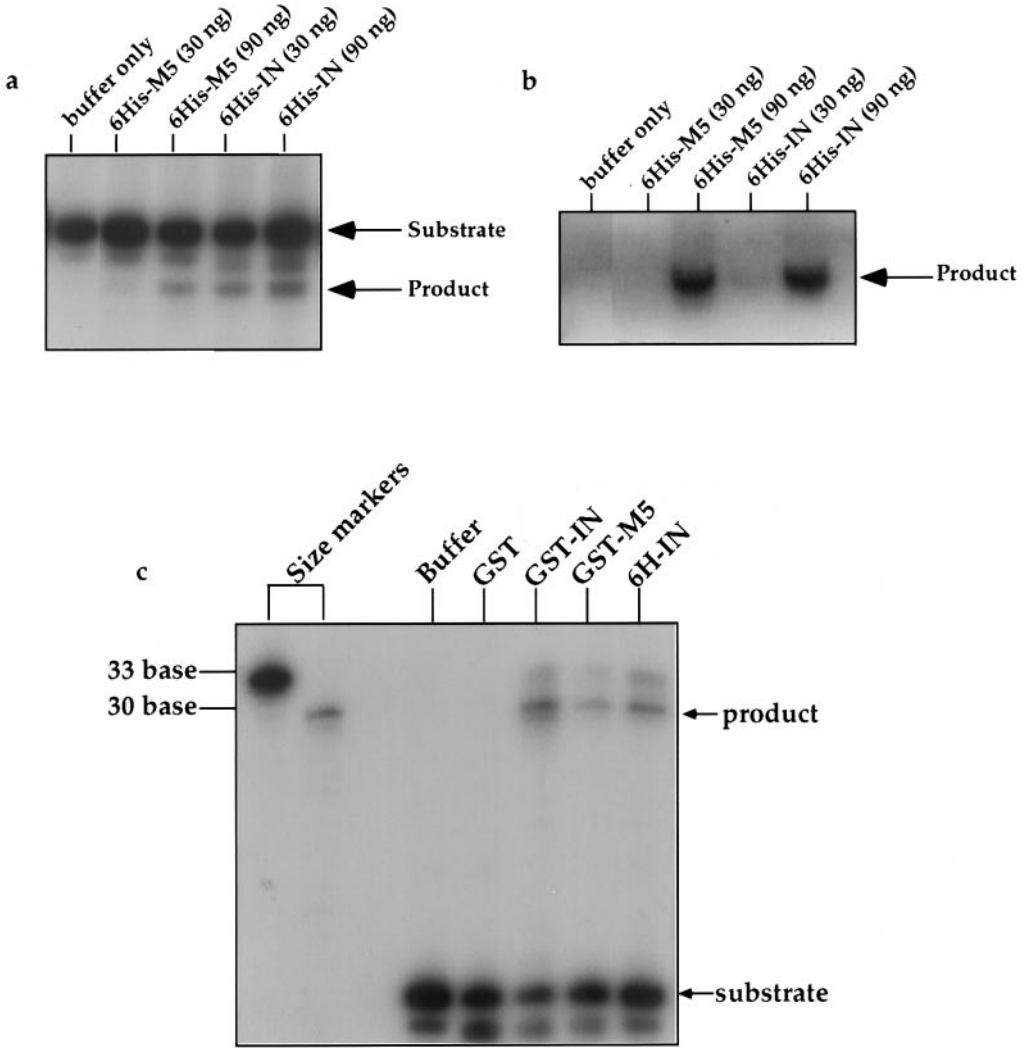

FIG. 4. Activities of V260E mutant *in vitro*. (a) 3′ processing activity assayed using labeled oligonucleotide substrate. The 18-mer was incubated in reaction buffer with 30 ng (lanes 2 and 4) or 90 ng (lanes 3 and 5) of either 6H-M5 or 6H-IN proteins, and the products were analyzed by polyacrylamide gel electrophoresis followed by autoradiography. The position of migration of products missing 2 nt at the 3′ end is indicated at the right. (b) DNA joining activity assayed using labeled oligonucleotide substrate and plasmid DNA as target and 90 ng each of 6H-IN and 6H-M5. Reaction products were detected by electrophoresis followed by autoradiography. The position of migration of circular product DNA is indicated. (c) Disintegration activity assayed using Y substrate and GST–IN (300 ng), GST–M5 (100 ng), and 6H-IN (90 ng) proteins per 30-μl reaction. A 16-mer oligonucleotide as substrate is converted to a 30-nt product. The position of marker oligonucleotides is indicated at the left and that of the substrate and the major product at the right. The protein preparations were as in a and b.

6A and 6B). The mature Gag and Pol cleavage products were detected in the medium after transfection with both wild-type and M5 DNAs. The M5 mutant showed about a three- to fivefold reduction in the yield of all the proteins. The relative proportions of each protein in the mutant virus, however, were comparable to those of wild type (Fig. 6A). To determine whether the reverse-transcriptase (RT) activity of the mutant virus was affected, wild-type and mutant virions were isolated from equal volumes of medium and assayed for RT activity (Goff *et al.,* 1981). The M5 mutant virus demonstrated about three to five times less RT activity than the wild type (data not shown), consistent with the reduced amounts of virion particles. This indicated that the RT activity per virion of the mutant was unaffected. Thus, the M5 viral proteins, including IN, are produced, processed, and incorporated into virion particles, with only a modest effect on the overall efficiency of the late stages of virus replication.

To determine whether the M5 mutation affected the infectivity of the virus, Jurkat cells were either infected with equal volumes of wild-type and mutant virion particles harvested from transfected COS7 cells or transfected with proviral DNA by the DEAE–dextran method (Reicin *et al.,* 1995). In both infection and transfection, only integration-competent virus can spread in the culture. Viral propagation was monitored by assaying the RT activity of the supernatant of the infected cells every 2–3 days (Fig. 7). RT activity of the wild type peaked 15 days

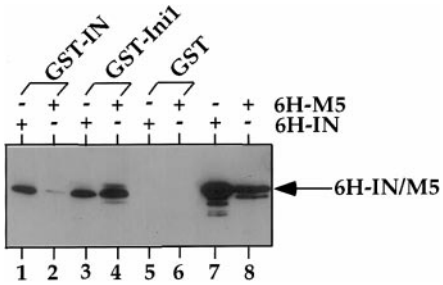

FIG. 5. *In vitro* binding studies of mutant and wild-type IN proteins. Glutathione beads were loaded with GST–IN, GST–Ini1, or GST alone as indicated and incubated with bacterial lysates containing hexa-histidine-tagged mutant (6H-M5) or wild-type (6H-IN) integrase. The beads were washed and the bound proteins were recovered and analyzed by electrophoresis and Western blot with anti-hexa-His antibodies (lanes 1–6). Aliquots of the lysates were analyzed directly to monitor input protein (lanes 7 and 8). The position of migration of the IN proteins is indicated. The levels of proteins were estimated by densitometry.

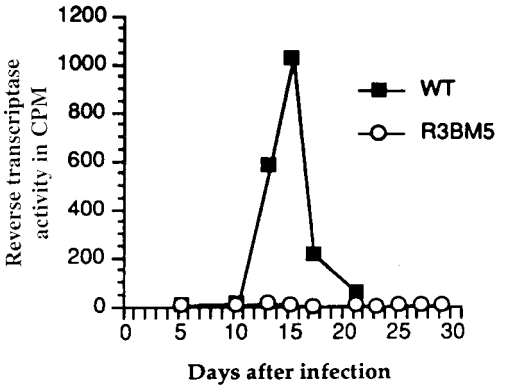

FIG. 7. Infectivity of the mutant virus M5. Virion particles harvested from the supernatants of transfected COS7 cells were used to infect Jurkat cells. Viral replication was monitored by measuring RT activity in the culture supernatant at various times. Each RT value represents the amount of activity in 1.5 μl of culture supernatant.

postinfection, whereas that of the mutant showed only background levels of activity throughout the time course of the experiment. These results indicate that the mutant virus is not capable of sustaining a spreading infection in culture.

To determine which stage of the viral life cycle was affected by the mutation, the ability of the M5 virus to carry out DNA synthesis *in vivo* was assessed. Circular forms of the retroviral genome containing one or two LTRs are produced only after the complete synthesis of viral DNA and its transport to the nucleus (Brown *et al.,*

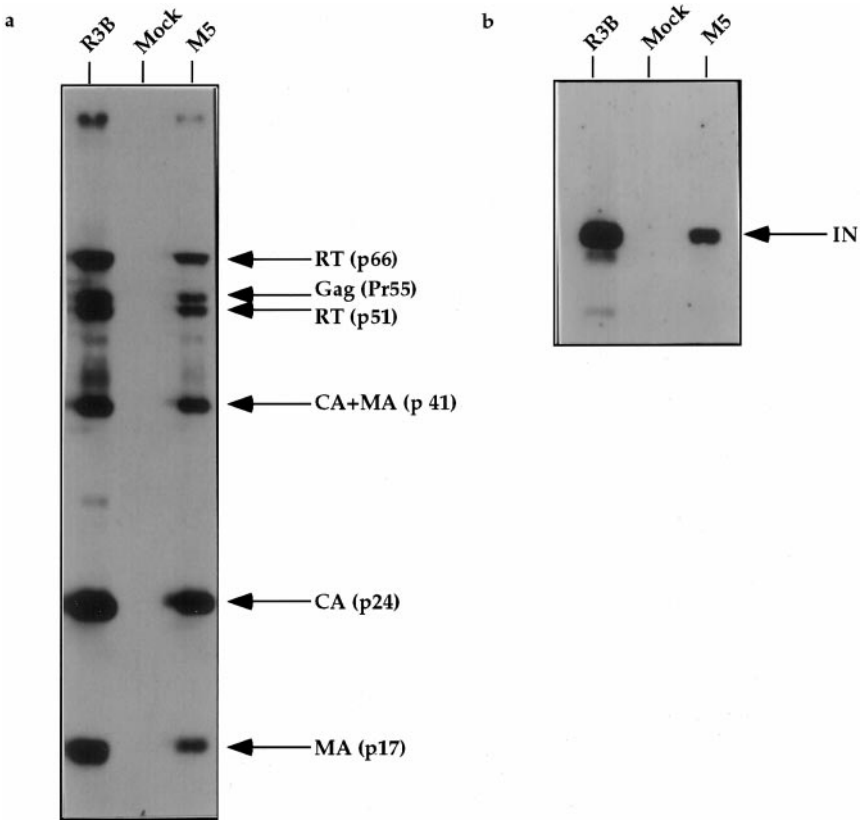

FIG. 6. Western analysis of viral proteins in the wild-type and mutant virion particles. Virion-associated proteins released by COS7 cells after transfection with wild-type and mutant proviral DNA were separated by electrophoresis on an SDS–12% polyacrylamide gel, transferred to nitrocellulose, and probed with HIV-1 antiserum (a) or monoclonal anti-IN antibody (b). Gag and Pol proteins are indicated by arrows. Lanes R3B, wild-type HIV-1 virus; M5, R3B virus with V260E substitution in IN; mock, mock-transfected cells.

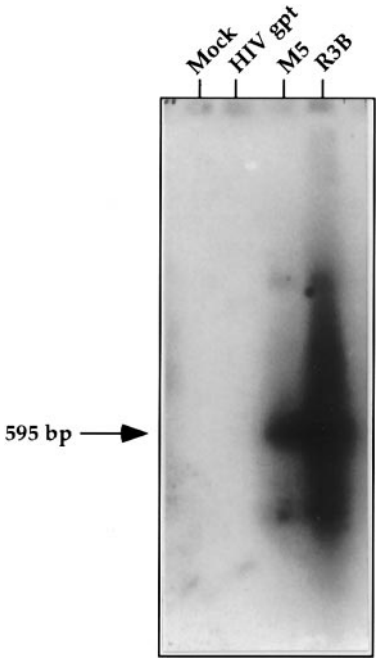

FIG. 8. PCR analysis of unintegrated circular viral DNA. Cloned viral DNA was used to generate virus in COS7 cells, the virus was applied to Jurkat cells, and the DNA isolated from these cells was subjected to PCR amplification. The product DNAs were displayed by electrophoresis on an agarose gel and subjected to Southern analysis. The position of the 595-bp LTR-LTR fragment hybridizing to the probe is indicated. Mock, mock-infected cells. HIV-gpt, an env-negative proviral DNA control. M5, mutant M5 proviral DNA. R3B, the wild-type proviral DNA.

1987). Thus the formation of the LTR–LTR junction of the two-LTR circle can be used as a marker of complete cDNA synthesis and nuclear entry of the virus preintegration complex. Equal volumes of pelleted wild-type and mutant virions harvested from COS7 transfected cells were used to infect Jurkat cells. Twenty-four hours postinfection, the cells were lysed and total cellular DNA was extracted. PCR analysis was performed on this DNA with primers specific for the LTR–LTR junction (Bukrinsky *et al.,* 1992). The PCR products were separated by electrophoresis and detected by Southern analysis using a radiolabeled third oligonucleotide as probe. DNA corresponding to the two-LTR junction was detected in both wild-type and mutant infected Jurkat cells (Fig. 8). Although the PCR–Southern assay is not strictly quantitative, estimating the amount of circular DNA detected in the mutant infected Jurkat cells suggested a reduction in the yield that was roughly consistent with the reduced virion particles present in the preparation. These results indicate that the mutant virus assembles and releases virion particles, reverse-transcribes the RNA into DNA, and transports the preintegration complex to the nucleus of the infected cell without gross defects. Thus, the defect in the M5 mutant most likely occurs during the formation of the provirus DNA itself.

DISCUSSION

A number of *in vitro* mutagenesis studies of IN have helped to define residues essential for its various enzymatic activities (Cannon *et al.,* 1994; Drelich *et al.,* 1992; Engelman and Craigie, 1992; Kulkosky *et al.,* 1992; Leavitt *et al.,* 1993; Shin *et al.,* 1994; Wiskerchen and Muesing, 1995). We have used the yeast two-hybrid system to screen specifically for integrase mutants that were defective in homodimerization, identifying one mutation, M5, that resulted in an interaction-negative phenotype. The M5 mutant IN did not interact either with itself or with wild-type IN in any combination in yeast. However, the mutant protein was able to interact with INI1 (hSNF5), the integrase interactor 1, indicating that the mutant was still capable of heteromeric interactions. This test and Western blot analysis indicated that the mutant was expressed stably in the yeast cells and was folded and localized properly in the yeast nucleus to permit interaction.

Our initial screen to isolate interaction-negative mutants of IN resulted in only one true mutant. Modification and further improvement of this method should facilitate large-scale screening: in particular, early screening of mutants for the ability to produce stable protein by Western blot should eliminate the isolation of mutants unable to produce stable, full-length proteins. Counterscreens, in which an interaction with a control protein is tested and selected for, can also be used to eliminate uninteresting mutants (Brachmann and Boeke, 1997; Vidal *et al.,* 1996; White, 1996).

The general localization of the M5 mutation to the C-terminal domain is intriguing since this domain can be deleted without loss of IN–IN dimerization in many assays. Thus, one possibility is that M5 might actively destabilize dimerization, in the sense that it might block dimerization more severely than the mere absence of the C-terminus. Alternatively, the M5 mutation may affect a particular dimer interface needed specifically for the multimerization of full-length IN into a larger structure. The active form of the protein is probably tetrameric, and in such a structure there can be two distinct dimer interfaces. The C-terminal region may be involved in only one of these contacts, and M5 may disrupt only this particular interaction. It is quite possible that the yeast system is monitoring the formation of such a larger oligomer; the GAL4DB–IN protein is likely to form dimers on the DNA and to then interact with a dimeric GAL4AD–IN. The *in vitro* binding assay may also monitor such an interaction, as the GST–IN protein on beads may exist as a dimer that presents the same surface to the soluble IN proteins in the lysate. Interestingly, when the binding experiment was reversed—with 6H-IN or 6H-M5 proteins bound to Ni-NTA beads and GST–IN in solution—no difference was observed between wild-type and mutant proteins in their ability to bind to IN (data not shown). Thus, in some

settings, the mutant protein may retain at least one functional interaction surface.

The M5 interaction-negative mutant recovered in this screen proved to contain a valine to glutamic acid substitution near the C-terminus of integrase. The simplest explanation is that the new residue might directly block a contact needed for oligomerization of IN. The structure of a dimer of the C-terminal domain as determined by NMR (Eijkelenboon *et al.,* 1995; Lodi *et al.,* 1995) is consistent with this notion. The domain forms a five-stranded β-barrel structure in solution, with residues in strands 2, 3, and 4 forming the interface of the dimer. The V260 residue lies in β strand 4, and though not directly bonded to the other subunit, it is immediately adjacent to a residue (V259) directly in contact with a residue in the other subunit (I257). The side chain of the very next residue, K258, is critical for DNA binding and points upward from a saddle-shaped groove in the dimer. The activity and oligomerization properties of several mutants of IN with substitutions of residues within the C-terminal domain have recently been reported (Puras Lutzke and Plasterk, 1998). Interestingly, the V260E mutation did not affect the DNA binding activity of the C-terminal fragment of HIV-1 IN (aa 220–270), suggesting that this mutation does not grossly disrupt the folding of the C-terminal domain (Puras Lutzke and Plasterk, 1998). Although preparations of the V260E mutant protein were not suitable for determining the oligomeric state, gel filtration analysis indicated that mutant INs with substitution of either of the nearby residues L241A or L242A were modestly impaired for tetramerization and not dimerization (Puras Lutzke and Plasterk, 1998). Thus, these results are consistent with the idea that the C-terminal domain might be important for tetramerization, rather than dimerization, of the full-length IN.

Assays of the activities of the M5 IN protein *in vitro* indicated that the mutation reduced the efficiency of 3′ processing and joining about 3- to 5-fold, but had no effect on disintegration. It should be noted that the use of IN fusions in these assays could affect the total activities and could even mask some inherent defects in the native protein. Similar studies of the V260E mutant in another laboratory recently showed a somewhat larger effect on processing and joining—roughly a 10-fold reduction—and also showed no effect on disintegration in one assay (Puras Lutzke and Plasterk, 1998). The small quantitative difference in the effects seen by the two laboratories may be due to differences in the methods of preparation of the proteins or the assays. The results suggest to us that the mutant protein can fold well enough to exhibit at least minimal nonconcerted integration and disintegration reactions *in vitro.* However, we cannot rule out the possibility that the mutation may destabilize the native state of the protein to some extent, and there may be some defects beyond those that can be attributed solely to the block to oligomerization.

The M5 mutation in the full-length genome of HIV-1 had profound effects on HIV-1 replication. Western analysis of the proteins in the virus particles indicates that the mutation had only modest effects on the production, processing, or incorporation of any of the viral proteins, including IN. PCR analysis of total DNA isolated from Jurkat cells 24 h postinfection with mutant virus demonstrated the presence of significant amounts of two-LTR circle junctions, indicating that the viral DNA was synthesized and successfully entered the nucleus. This suggests that the major defect was in viral integration *in vivo* and indicates that proper oligomerization of IN is essential for viral replication. This finding suggests that disrupting the oligomeric state of IN would be an effective antiviral strategy: drugs and dominant negative inhibitors that specifically affect the oligomerization of IN may be useful therapeutically.

MATERIALS AND METHODS

Yeast and bacterial strains

Standard cloning methods were carried out in strain DH5α. Mutagenesis of DNAs was performed in LE30 (a mutator strain with *mutD5* mutation; Prasad *et al.,* 1991); plasmids were methylated by propagation in CC114 [del (ara,leu)7697, lacZ-amY14, galU, galK, hsdR$^-$, *hsdM*$^+$, strAr, rifr, argE-am, srl::Tn10, recA1; Lobel and Goff, 1984]. BL31 (DE3), carrying an integrated copy of the T7 polymerase gene, was used to express wild-type and mutant recombinant IN proteins. Two-hybrid tests were performed in *Saccharomyces cerevisiae* GGY1:171 (*gal4 gal80 ura3 his3 leu2;* Gill and Ptashne, 1987).

Construction of plasmid clones

Plasmids expressing GAL4IN fusion proteins, pMAI (GAL4DB fused to IN) and pGADI (GAL4AC fused to IN), have been previously described (Kalpana and Goff, 1993). A T7 expression vector, pT7fl1 (gift of Ron Swanstrom), was modified to facilitate expression of mutant INs in *E. coli.* The DNA was cleaved with *Nde*I, the single-stranded overhangs were filled in using the Klenow fragment of DNA *PolI,* and the blunt ends were ligated to generate plasmid p12K. DNA fragments containing IN sequences were excised from yeast plasmids by *Bam*HI cleavage and transferred to p12K to generate expression clones p12KM5 and p12KIN. To generate clones pQE32-IN and pQE32-M5 for the expression of Hexahistidine (6H) tagged IN proteins in bacteria, *Bam*HI and *Sal*I fragments encoding the wild-type and mutant IN sequences were isolated from the yeast plasmids and ligated to the bacterial expression vector pQE32 (Qiagen), digested with the same restriction enzymes. To generate clones pGST-M5 and pGST-IN for the expression of GST fusion proteins, a *Bam*HI fragment of IN and a *Bam*HI to *Sal*I fragment of M5 were cloned into plasmid pGEX3X.

The M5 mutation was transferred to a molecular clone of the HIV-1 provirus in two steps. First, a *Bsp*MI fragment (corresponding to bp 4317–5045 of the HxBC.2 virus) containing the M5 mutation was isolated from the plasmid p12KM5 and was used to replace the corresponding wild-type fragment in the plasmid pBSHIV. Plasmid pBSHIV is a Bluescript plasmid containing the *Spe*I–*Sal*I fragment (corresponding to bp 1506 to 5785) of HxBC.2. Second, the *Spe*I to *Sal*I fragment containing the M5 mutation was used to replace the corresponding region of the HIV-1 proviral clone R73/BH10 (Aldovini and Feinberg, 1990).

Mutagenesis of integrase

To generate a library of IN variants, plasmid pMAI was first introduced into CC114 to methylate the plasmid and then reisolated and used to transform the restriction positive (*hsdR*$^+$) mutator strain LE30. To ensure that the host's mutator phenotype was functioning, the rate of mutagenesis of a test plasmid in a number of independent cultures of LE30 was determined as follows. LE30 bacteria were streaked onto plates containing minimal medium (M9) supplemented with casamino acids but without thymidine; this medium limits the mutagenesis of LE30 genomic DNA. Competent cells were prepared from several individual colonies picked from the M9 plates and stored frozen. A test plasmid containing the full-length *lacZ* gene (pBW5; gift of LaDonne Schulman) was introduced into aliquots from each batch of competent LE30 cells. Plasmid DNA was prepared from each pool of transformants and introduced into a *lacZ*^{am} strain of *E. coli* (CC114), and the percentage of mutant *lacZ*$^-$ plasmids was estimated by scoring blue and white transformants on X-gal plates. LE30 mutator subclones typically induced 1–5% *lacZ*$^-$ mutants. The batch of competent cells with the desired mutation rate was then transformed with the plasmid pMAI. Approximately 20 LE30 transformants carrying the pMAI plasmid were inoculated into LB and incubated overnight at 37°C to induce mutations. Plasmid DNA prepared from this culture served as the library of GAL4–IN mutants.

Expression and purification of IN proteins

GST–IN and GST–M5 proteins. Cultures of *E. coli* strain HB101 harboring either pGST–IN or pGST–M5 plasmids were used to produce the corresponding fusion proteins. Bacterial cultures were induced by the addition of 1 mM IPTG. The washed cell pellet was resuspended in lysis buffer (20 mM Tris–Cl, pH 8.0, 1 M NaCl, 1 mM EDTA, 1 mM PMSF) and lysed by passage through a French press twice at approximately 1000 psi pressure. The lysate was passed through an 18-gauge needle and clarified by centrifugation. The supernatant was loaded onto a glutathione–agarose column equilibrated with wash buffer [20 mM Tris–Cl, pH 8.0, 0.5 M NaCl, 1 mM EDTA, 0.5% IGEPAL (a nonionic detergent; Sigma), and 1 mM PMSF]. The column was washed extensively with wash buffer and the bound proteins were eluted with elution buffer (50 mM HEPES, pH 8.0, 20 mM glutathione, 0.4 M NaCl, 0.1 M LiCl, 1 mM EDTA, 0.5% IGEPAL, and 1 mM PMSF). The eluted proteins were dialyzed against several volumes of dialysis buffer (50 mM HEPES, pH 8.0, 0.5 M NaCl, 0.1 mM EDTA, 20% glycerol, 1% thiodiglycol, 1 mM PMSF) and stored at −70°C.

6H-IN and 6H-M5 proteins. Wild-type and mutant IN proteins were isolated as described previously (Kalpana *et al.,* 1994) by FPLC on Ni-NTA Sepharose columns (Pharmacia). The level of expression of 6H-M5 protein was two- to fivefold reduced compared to the level of expression of 6H-IN. The bound proteins were washed and then eluted using a gradient of 40–800 mM imidazole, and fractions containing IN and M5 were collected, pooled, and dialyzed against buffer containing 20 mM HEPES, pH 7.2, 1 mM DTT, 1 mM EDTA, 0.5 M NaCl, 20% glycerol, and 1 mM PMSF.

In vitro enzyme assays of GST fusion proteins

3′ processing and DNA joining of 6H-IN and 6H-M5 fusion proteins, and disintegration activities of GST-IN and GST-M5 fusion proteins, were assayed essentially as described (Chow *et al.,* 1992; Engelman *et al.,* 1991) using radiolabeled substrates. Joining activity was studied using pBluescript plasmid as target DNA and preprocessed oligonucleotide substrates.

Viral infectivity studies

COS7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Jurkat cells were maintained in RPMI 60 medium with 10% fetal calf serum. Both wild-type and mutant viral stocks were prepared by transfecting COS7 cells with 20 μg of viral DNA per 2–4 $\times$ 10^6 cells by the calcium phosphate precipitation method (Graham and van der Eb, 1973). Sixty hours posttransfection, culture supernatants were harvested, filtered, and concentrated by centrifugation through a 25% sucrose cushion in 10 mM Tris–HCl, 10 mM NaCl, and 1 mM EDTA (TNE) at 4°C for 2 h at 10,000 *g* (Reicin *et al.,* 1995). Infectivity was tested either by infecting Jurkat cells with virions collected from COS7 supernatants or by transfecting Jurkat cells with 1 μg of R3B (wild type) or R3BM5 (mutant) proviral DNA by the DEAE–dextran method (McCutchan and Pagano, 1968). To test for the spread of virus throughout the cell culture, viral supernatants were harvested every 2 to 3 days and assayed for RT activity (Goff *et al.,* 1981).

Western analysis

Virion particles released from COS7 cells were concentrated as above and were lysed by the addition of

Triton X-100 as previously described (Reicin *et al.,* 1995). The viral protein samples were separated by electrophoresis on an SDS–polyacrylamide gel, transferred to nitrocellulose, and probed with either HIV-1 infected patient antiserum 256 (kindly provided by John Moore) or mouse anti-HIV-1 IN (gift of Dag Helland). Proteins were visualized by the enhanced chemiluminescence method as prescribed by the manufacturer (Amersham).

Isolation of DNA from infected cells and PCR analysis

Virions were harvested and pelleted from transfected COS7 cell supernatants at 60 h posttransfection as described above. The pelleted virions were resuspended in 200 μl of RPMI and placed at 4°C overnight. The virions were treated with 10 U of DNase A (Promega) for 20 min at 37°C after the addition of $MgCl_2$. Jurkat cells (6×10^6) in 2.5 ml of RPMI containing 10% fetal calf serum and 0.8 μg/ml of polybrene were added to the DNase A treated virions. The cultures were incubated at 37°C for 2 h with gentle shaking every half-hour, diluted 10-fold with fresh medium (lacking polybrene), and placed at 37°C. Twenty-four hours postinfection, the cells were harvested and total genomic DNA was prepared (Bukrinsky *et al.,* 1992). An aliquot of the total genomic DNA was subjected to PCR analysis using primers corresponding to the U5 region of the plus strand (nucleotides 9650–9669) and the R region of the minus strand (nucleotides 9591–9610). Nucleotide positions of the oligonucleotide primers are according to the sequence of Ratner *et al.* (1985). The PCR products were subjected to agarose gel electrophoresis and Southern analysis using a third oligonucleotide corresponding to nucleotides 9427–9446 as probe. This analysis detects a 595-bp DNA fragment corresponding to the two-LTR junction of the circular viral DNA (Bukrinsky *et al.,* 1992).

Sequence analysis

Primers ZG1 (5′CCTGCCCTGTTTCTGC3′), ZG2 (5′GCCTGATCTCTTACCTG3′), IN3 (5′GGAGCTTTGCTGGTCC3′), and IN4 (5′CCCTGAAACATACATATGGTG3′) were used to sequence the entire length of mutant and wild-type integrases in clones p12KM5 and p12KIN, respectively. Sequences were determined by automated fluorescence sequencing methods. The presence of the mutation in clone pQE32-M5 was confirmed by sequencing the plasmids using the IN3 primer.

In vitro binding assays to monitor the association of mutant and wild-type HIV-1 IN

To determine the ability of the mutant IN protein to associate with wild-type IN *in vitro,* a simple binding assay was used. GST fusion proteins were expressed and recovered on glutathione beads as described above. The beads were washed and incubated with bacterial lysates containing 6H-IN proteins as described previously (Kalpana *et al.,* 1994). After binding, the beads were washed three times, and the bound proteins were eluted by boiling in sample buffer containing SDS and DTT and subjected to SDS—polyacrylamide gel electrophoresis. The 6H-IN proteins were detected by immunoblotting with polyclonal anti-hexa-His antibodies by a chemiluminescence detection method (Pierce).

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