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Tethering Human Immunodeficiency Virus Type 1 Preintegration Complexes to Target DNA Promotes Integration at Nearby Sites

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Integration of retroviral cDNA in vivo is normally not sequence specific with respect to the integration target DNA. We have been investigating methods for directing the integration of retroviral DNA to predetermined sites, with the dual goal of understanding potential mechanisms governing normal site selection and developing possible methods for gene therapy. To this end, we have fused retroviral integrase enzymes to sequence-specific DNA-binding domains and investigated target site selection by the resulting proteins. In a previous study, we purified and analyzed a fusion protein composed of human immunodeficiency virus integrase linked to the DNA-binding domain of λ repressor. This fusion could direct selective integration in vitro into target DNA containing λ repressor binding sites. Here we investigate the properties of a fusion integrase in the context of a human immunodeficiency virus provirus. We used a fusion of integrase to the DNA binding domain of the zinc finger protein zif268 (IN-zif). Initially we found that the fusion was highly detrimental to replication as measured by the multinuclear activation of a galactosidase indicator (MAGI) assay for infected centers. However, we found that viruses containing mixtures of wild-type integrase and IN-zif were infectious. We prepared preintegration complexes from cells infected with these viruses and found that such complexes directed increased integration near zif268 recognition sites.

Retroviral vectors are widely used in gene therapy, in part because the viral integration system attaches viral cDNA to host DNA with precise covalent bonds. However, integration also creates a possible hazard. Since integration is not sequence specific, the inserted viral DNA may potentially inactivate or inappropriately activate host genes. Many studies with vertebrate systems have established that integration of retroviral DNA can result in inactivation or ectopic activation of cellular genes, thereby causing diseases (for a review, see reference 15). Shiramizu et al. have described examples of non-Hodgkin's lymphomas from patients with late-stage AIDS in which integration of human immunodeficiency virus (HIV) apparently activated the c-*fps*/c-*fes* oncogene (39).

In an effort to develop retroviral derivatives capable of integrating into predetermined locations, we have been investigating the activities of fusion proteins composed of the HIV-1 integrase fused to sequence-specific DNA binding domains (6, 7, 32). Integrase protein is essential for integration of HIV cDNA after infection. In vitro, purified integrase is capable of forming covalent bonds between a model viral DNA and model target DNA (8, 9, 16, 28; for a recent review, see reference 23).

In a previous study, we fused HIV-1 integrase to the DNA binding domain of λ repressor and purified the protein after overexpression in *Escherichia coli*. The activity of the fusion (named λR-IN) was compared with that of wild-type integrase in assays in vitro. Wild-type HIV-1 integrase was found to direct integration into DNA targets with similar frequencies regardless of the presence or absence of λ operators (binding sites for λ repressor). The λR-IN fusion, however, directed integration preferentially to targets containing λ operators. Integration directed by λR-IN was excluded from the operator sites, as expected if λR-IN was bound to those sites. Integration was found to take place preferentially near the λ operators, at sites on the same face of the DNA helix as the λ operators. This indicated that λR-IN bound to DNA likely captured target DNA by looping out the intervening sequences. Integration on the opposite face of the DNA helix was probably disfavored due to the torsional rigidity of DNA. These data establish that tethering an integrase protein to target DNA can be sufficient to direct integration preferentially to local DNA sites (6).

More recently, two other groups have also made fusions of integrases to sequence-specific DNA binding domains. These studies established that various combinations of integrases and binding domains can be functional in vitro and that fusions can be made to either end of integrase proteins (25, 29).

To develop retroviral derivatives that carry out site-specific integration, functional integrase fusions must be introduced into virions. This requires the assembly of gag-pol polyproteins containing integrase fusions into particles in virus-producing cells. Following budding and infection of target cells, the fusion integrase must become associated correctly with the viral cDNA following reverse transcription and must participate in integration.

In vivo, the viral cDNA is found in association with integrase and other proteins in large complexes named preintegration complexes. Such complexes can be obtained from retrovirus-infected cells and used as a source of integration activity in vitro (5, 20, 24). A comparison of in vitro reactions involving preintegration complexes with reactions involving purified integrase indicates that the former more closely model integration in vivo (see reference 23 for a discussion). For example, preintegration complexes carry out the coordinated joining of both ends of the unintegrated linear viral cDNA (5, 20, 24) whereas purified integrase carries out a partial reaction in which only one DNA end is joined efficiently (9). Preintegration complexes and purified integrase also differ in their re-

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sponse to integrase inhibitors (22, 23). Previous studies of fusions of integrase to DNA binding domains have examined only the activities of fusions purified from *E. coli* after overexpression (6, 7, 25, 29, 32).

We have examined the activities of a fusion of integrase to the zinc finger protein zif268 (IN-zif) in virions and preintegration complexes. We found initially that virions harboring IN-zif were greatly impaired for replication. However, virions containing mixtures of wild-type integrase and IN-zif were competent to carry out reverse transcription and integration. Preintegration complexes isolated from cells infected with such virions showed increased integration in vitro near zif268 recognition sites.

MATERIALS AND METHODS

Construction of a provirus encoding a fusion of integrase to the DNA binding domain of zif268. The R9′-INzif provirus (see Fig. 1) was constructed by first introducing new restriction sites into the DNA encoding the carboxyl terminus of integrase and then transferring sequences encoding the zif268 DNA binding domain. The HIV provirus R9 is a hybrid of pNL4-3 (bases 712 to 8473) and HXB2 (bases 1 to 711 and bases 8474 to 9709) (a gift of D. Trono). Plasmid pMM101 consists of a 3.7-kb *Apa*I-*Eco*RI fragment of R9 cloned into pBluescript II SK+. *Eco*RI and *Cla*I sites were appended to the 3′ end of the integrase coding sequence by PCR with primers FB156 (5′-GGAATTGGAGGTTTTATCAAAGTA-3′, corresponding to nucleotides 2394 to 2417 of pNL4-3) and MM101 (5′-GGCGAATTCCTAATCGATATCCTCATCCTGTCTACTTGCCACACA-3′, where the appended nucleotides are underlined and the rest are complementary to nucleotides 5090 to 5064 of pNL4-3). The 2.7-kb PCR product was digested with *Eco*RI and *Bcl*I and cloned into R9 digested with the same enzymes. The resulting clone, pMM102, contains unique *Cla*I and *Eco*RI sites just 3′ of the integrase coding sequence, encodes an integrase protein that contains two extra amino acids (Ile and Asp) at its carboxyl terminus, and has a deletion of 648 bp (5091 to 5739 in NL4-3) encoding parts of vif and vpr. A DNA fragment encoding an influenza virus hemagglutinin epitope and a hexahistidine tract separated by a *Sma*I site was created by annealing two complementary oligonucleotides, MM102 (5′-CGATTACCCATACGATGTTCCAGATTACGCTCCCGGGCATCATCACCATCATCATTGAG-3′) and MM103 (5′-AATTCTCAATGATGATGGTGATGATGCCCGGGAGCGTAATCTGGAACATCGTATGGGTAAT-3′). The resulting double-stranded oligonucleotide was cloned into pMM102 cleaved with *Cla*I and *Eco*RI to yield pMM103. Since early tests revealed that viruses encoded by this construction were impaired for replication, the sequences encoding nucleotides 5091 to 5739 were restored by amplifying the region from R9 by using primers of sequence 5′-CGGAAGCGAATTCGTGACACATGGAAAAGATTAGTAAAACAC-3′ and 5′-TAGGCTGACTTCCTGGATGCTTCCA-3′, cleaving the resulting PCR fragment with *Eco*RI, and ligating the fragment to pMM103 cleaved with *Eco*RI. A provirus with the new fragment in the wild-type orientation was named R9′ (encoded by plasmid pFB277). To form R9′-INzif, the region encoding the zif268 DNA binding domain (amino acids 333 to 424) was amplified from plasmid zif/268 (14) with primers of sequence 5′-CTCAGCGGATCGATCATGAACGCCCATATGCTTGCCCT-3′ and 5′-CGGTGCATCCCGGGTTATGCTTTCTTGTCCTTCTGTCTTAA-3′, the resulting PCR fragment was cleaved with *Cla*I and *Sma*I, and the fragment was ligated with R9′ cleaved with *Cla*I and *Sma*I.

Construction of integration target DNAs. Target DNA clones were constructed by ligating a duplex oligonucleotide consisting of bases 59 to 98 (see Fig. 4, top strand) and 79 to 116 (see Fig. 4, bottom strand) to pUC19 cleaved with *Bam*HI and *Eco*RI, yielding pFB298. A control target DNA was constructed in the same fashion except for point mutations in the zif sites (pFB299) (in each site, 5′-CGCCCACGC-3′ was changed to 5′-CGGCGACGG-3′). Target DNA fragments containing the zif sites were generated by digesting pFB298 or pFB299 with *Pvu*II, purified by electrophoresis, and recovered by binding to glass milk.

Analysis of infectivity of R9′-INzif virions. Proviral DNAs were expressed transiently in 293T cells by standard calcium phosphate transfection (1). One unit in Fig. 2 indicates 5 μg of DNA transfected. Culture supernatants were harvested 48 h after transfection and assayed for p24 antigen concentration by enzyme-linked immunosorbent assay (DuPont) and infectious units with P4-2 indicator cells (11). Western blot analysis was performed essentially as described previously (1) with a rabbit polyclonal antibody raised against HIV-1 integrase purified from an *E. coli* expression system.

Preparation of preintegration complexes. Preintegration complexes were generated, starting with cloned proviral DNA, by coculturing 293T cells expressing R9′ or R9′+R9′-INzif virus with SupT1 target cells. Two days after transfection, 293T cells (1×10^7 cells in 10-cm dishes) were mixed with SupT1 cells (4×10^7 cells per dish). After incubation for 7 h, the medium was removed and syncytia were washed once in buffer K and then permeabilized in buffer K plus 0.025% Brij 96 (1 ml per dish; buffer K contains 20 mM HEPES [pH 7.3], 150 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 10 U of aprotinin per ml, 0.1 mM AEBSF, and 1 μM pepstatin). After 10 min at room temperature, a cytoplasmic extract was prepared by centrifugation at $1,000 \times g$ for 3 min and then the supernatant was clarified by centrifugation at $8,000 \times g$ for 3 min. Aliquots (1.8 ml) of extract were then layered over 0.2-ml pads of buffer K containing 20% sucrose and subjected to centrifugation for 30 min at 14,000 rpm in a tabletop centrifuge (Eppendorf). The supernatant was then removed, and the pellets were resuspended in 200 μl of buffer K.

Wild-type preintegration complexes were also generated by coculture of SupT1 target cells with MoltIIIB HIV-1 producer cells as described previously (24).

Integration reactions. Integration reactions were carried out by adding the target DNA fragment (18 nM) to 100 μl of preintegration complexes in buffer K. Reaction mixtures were preincubated on ice for 20 min, and then the reactions were started by transferring the mixture to 37°C for 15 min. The reactions were stopped by adding 1/8 volume of 5% sodium dodecyl sulfate plus 100 mM EDTA and 1/8 volume of proteinase K (10 mg/ml). After overnight incubation at 55°C, the reaction mixtures were extracted with a 1:1 phenol-chloroform, ethanol precipitated, and resuspended in 15 μl of TE. PCR analysis of integration sites was carried out as described previously (6). The sequences of amplification primers were 5′-TGTGGAAAATCTCTAGCA-3′ (viral end primer) 5′-AACAGCTATGACCATG-3′ (bottom strand primer), and 5′-GTTTTCCCAGTCACGAC-3′ (top strand primer). A 5-μl volume of sample was used for each amplification.

For reaction mixtures containing the zif268 DNA binding domain (a gift of Carl Pabo), target DNA (37 nM) and the indicated concentration of purified zif domain were preincubated in 100 μl of buffer K. The binding mixture was then added to 100 μl of preintegration complexes partially purified by spinning through a sucrose pad as described above.

RESULTS

Experimental plan. We selected the DNA binding domain of zif268 for use in integrase fusions for several reasons. The zif268 domain is small (93 amino acids [34]), potentially minimizing the disruption of virion architecture resulting from adding the new domain. zif268 binds DNA as a monomer (34), thus avoiding possible problems in multimerization of the DNA binding domain in the context of preintegration complexes in vivo. The IN-zif fusion was not degraded by proteolysis in virions, a problem we encountered with some other integrase fusions (data not shown). In addition, many derivatives of zif268 that have new DNA binding specificities have been created (12, 13, 17–19, 26, 38). This raises the hope that IN-zif fusions containing modified zif domains might eventually be used to direct integration into many different target DNA sites.

We formed a fusion of zif268 to the carboxyl terminus of integrase in the context of the R9′ HIV-1 provirus (Fig. 1). Virions produced under the direction of this DNA were tested for the ability to form infected centers in cell culture, and then preintegration complexes were prepared and analyzed. Studies of site selection by preintegration complexes were carried out by a PCR assay in vitro rather than assays in vivo because of the far greater convenience of analyzing site selection by this method.

Infectivity of viruses containing IN-zif fusions. DNA encoding wild-type HIV (R9′) or an isogenic derivative encoding the IN-zif fusion (R9′-INzif) was transfected into 293T cells. Supernatants were collected 2 days after transfection, and infectivity was measured with p4-2 indicator cells (11).

While wild-type R9′ virus yielded 760 infected centers per ng of capsid antigen, no significant infectivity could be detected for R9′-INzif virus (Fig. 2A). Since integrase acts as a multimer (21, 27, 40), we tested virions containing mixed wild-type and zif fusion integrase proteins in the hopes of restoring activity. We cotransfected wild-type R9′ DNA with R9′-INzif DNA and found that the virions produced (R9′+R9′-INzif; henceforth referred to as wild type + zif) displayed infectivity within a few fold of the wild-type values (Fig. 2A).

Virion proteins generated after such transfections were analyzed on Western blots probed with an anti-HIV-1 integrase antibody. A protein of the size expected for wild-type integrase

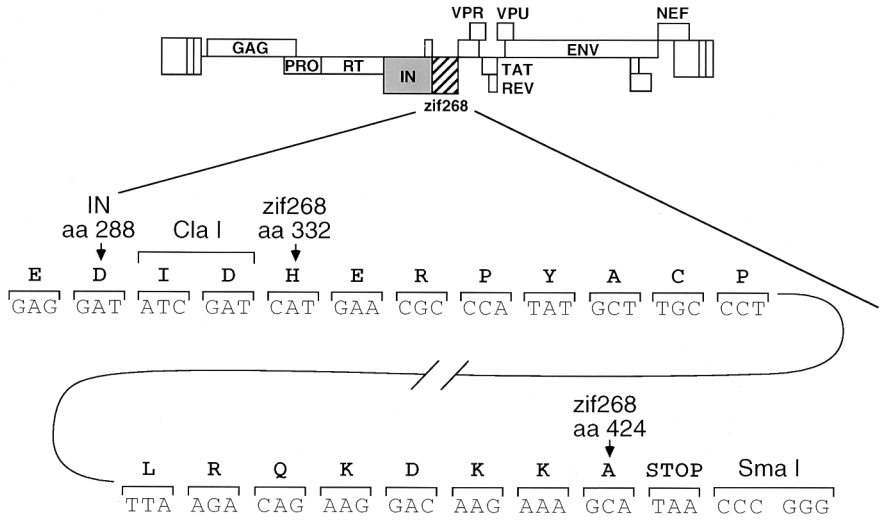

FIG. 1. Genetic map of an HIV provirus (R9′-INzif) encoding a fusion of integrase protein to the DNA binding domain of zif268. See the text for details. aa, amino acid.

was seen in samples from R9′ virions (Fig. 2B, lane 1, lower arrow). A protein of the size expected for IN-zif was seen in R9′-INzif virions (Fig. 2B, lane 2, upper arrow). Cells transfected with both DNAs (wild type + zif) yielded virions containing integrase proteins of both sizes (Fig. 2B, lanes 3 to 6).

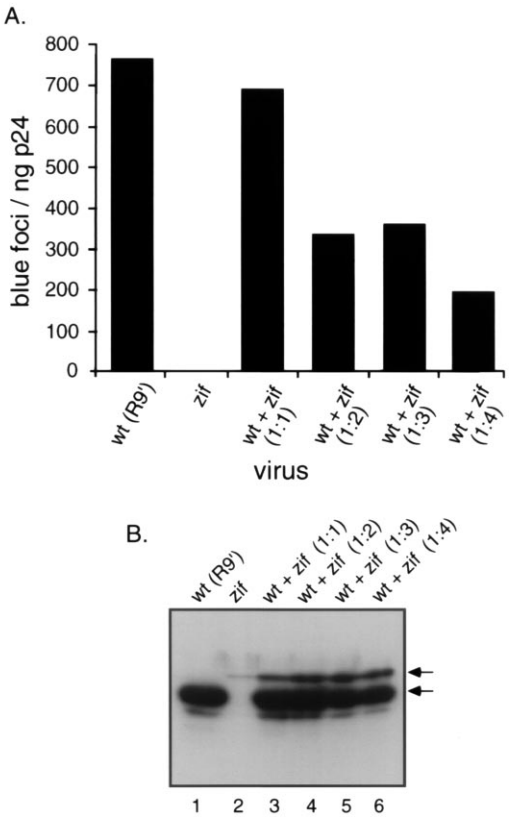

FIG. 2. Activity of an HIV provirus encoding integrase fused to the DNA binding domain of zif268. (A) Analysis of the infectivity of virions produced by transfection of wild-type (wt) (R9′), R9′-INzif, and mixed DNAs. Infected centers were scored by using p4-2 indicator cells (11). (B) Western blot analysis of integrase proteins in virions produced by transfection.

Preparation and analysis of preintegration complexes containing zif domains. To determine whether the new zif domain influenced integration site selection, we isolated and tested subviral preintegration complexes from cells infected with wild-type R9′ or wild-type + zif viruses. Lymphoid target cells (SupT1 cells) were infected by cocultivation with virus producer cells and preintegration complexes isolated from the cytoplasm 7 h later. Complexes were incubated with target DNA to allow integration (Fig. 3A, parts 1 and 2), and products were then assayed by PCR (parts 3 and 4) (6, 31, 37). One amplification primer bound to one end of the HIV cDNA, and the other bound to the target DNA near the zif sites. The viral primer was end labeled with ^{32}P. Amplification yielded a population of products of different lengths, where the length of each product was determined by the site of integration (Fig. 3A, part 3). The labeled PCR products were then separated by electrophoresis and visualized by autoradiography (Fig. 3A, part 4). Each band on the final gel reflected integration at a single phosphodiester in the target DNA. Separate target primers were used to monitor integration into the bottom strand (Fig. 3B, lanes 1 to 6) or top strand (lanes 7 to 12).

Integration carried out by preintegration complexes was compared with two different target DNAs. One target contained two recognition sites for zif268 embedded in a 339-bp DNA fragment. The second target was identical to the first except that each zif site contained three point mutations expected to block zif binding.

Reaction mixtures containing wild-type + zif preintegration complexes yielded different patterns of preferred sites when the target DNA contained intact zif binding sites (Fig. 3B, compare lanes 1 and 2 and lanes 7 and 8). Some positions of enhanced integration seen with wild-type + zif complexes are indicated in Fig. 3B by the rectangles beside lanes 2 and 8 and in Fig. 4 by the rectangles over the sequence. The effects of the IN-zif fusion were most pronounced near the zif binding sites and tapered off with increased distance away from these sites (Fig. 3B, lane 2, compare the 50-to-110 region to the 150-to-180 region; lane 8, compare the 50-to-130 region to the 180-to-260 region).

In contrast, when the zif sites contained mutations, the integration sites favored by wild-type and wild-type + zif complexes were indistinguishable (Fig. 3B, compare lanes 3 and 4

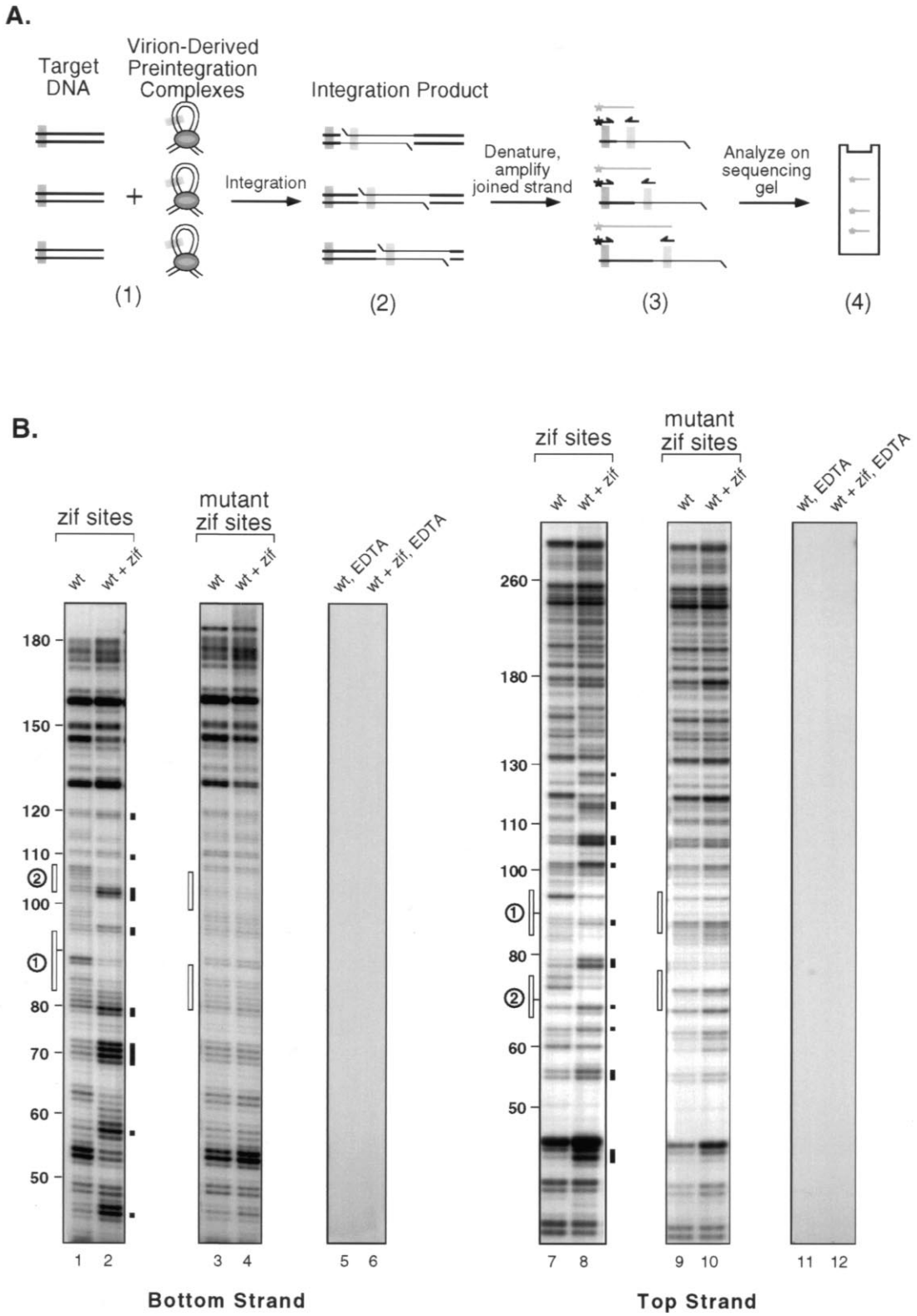

FIG. 3. Integration hot spots favored by preintegration complexes containing zif268 DNA binding domains linked to integrase. (A) Diagram of the PCR method used to analyze integration sites. Primer binding sites are shown as gray rectangles. An asterisk denotes a labeled DNA 5′ end. (B) Results of PCR integration assays of preintegration complexes derived from wild-type (R9′) virions or wild-type + zif (R9′+R9′-INzif) virions. Lanes 1 to 6 present the results of assays of integration in the bottom strand of the target DNA, and lanes 7 to 12 present the results of assays of integration in the top strand. The locations of the zif sites are marked by the open bars. Hot spots for integration by wild-type + zif preintegration complexes in the zif site target are marked by solid rectangles. The mutant zif sites contained three base changes expected to disrupt important contacts between zif protein and DNA (5′CGCCCACGC3′ changed to 5′CGGCGACGG3′) (34).

FIG. 4. Hot spots for integration by wild-type + zif (R9′+R9′-INzif) preintegration complexes in target DNA containing wild-type zif sites. Solid bars indicate the positions of enhanced integration. The zif binding sites are shaded.

and lanes 9 and 10). As a further control, reactions were carried out in the presence of 10 mM EDTA, which chelates Mg^{2+} ions essential for integration. No labeled PCR products were detected, indicating that the generation of PCR products depended on integration (Fig. 3B, lanes 5, 6, 11, and 12).

Effects of purified zif268 DNA binding domain on integration. A possible source of artifacts in the above study is the potential influence of free zif268 DNA binding domain on integration site selection. It is clear that DNA binding proteins that distort DNA can promote integration in or near the deformed part of the binding site (3, 4, 33, 35, 36). Although the X-ray structure of zif bound to its DNA site did not reveal prominent distortions (34) and preintegration complexes were partially purified prior to the assay, one might still wonder whether free zif domain bound to the target might distort DNA and promote integration.

To address this issue, purified zif DNA binding domain was added to integration reaction mixtures with wild-type preintegration complexes and the effects were assessed. Site selection in the region of the zif sites was monitored by the PCR-based assay described in Fig. 3A. The region of the zif-binding sites showed reduced integration signals in the presence of zif, as expected if a bound zif DNA binding domain blocked access of the preintegration complex to target DNA (Fig. 5, lanes 1 to 4,

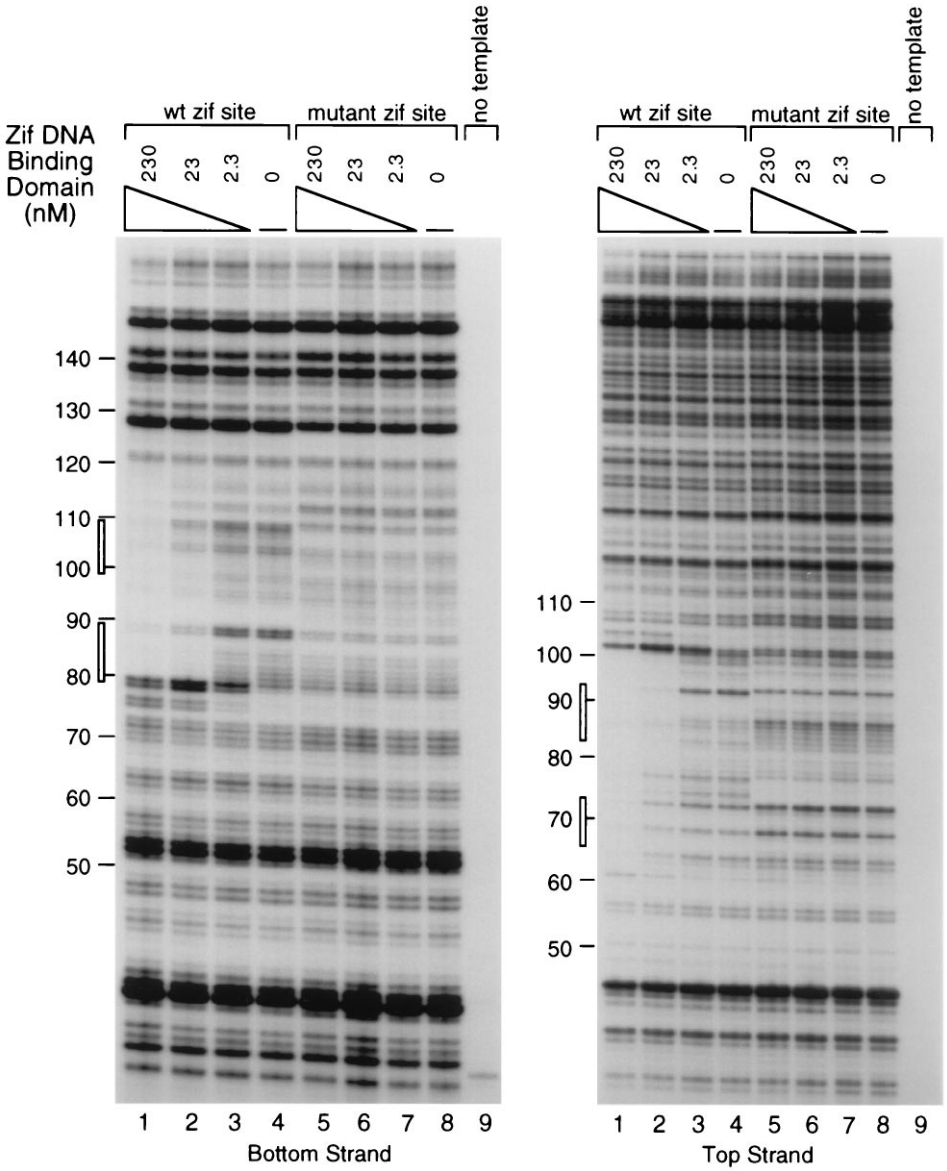

FIG. 5. Influence of purified zif268 DNA binding domain on integration site selection by wild-type preintegration complexes. The concentration of zif in each integration reaction mixture is shown above the gel lane. The integration targets used are exactly those used in the experiments in Fig. 3. The locations of the zif sites are marked by the open bars. Wild-type + zif complexes were not tested.

bottom and top strands). When the experiment was repeated with the target DNA containing mutant zif sites, no reduced integration was seen in the region of the zif sites (lanes 5 to 8, bottom and top strands). No integration was seen in the absence of added template DNA (lanes 9, bottom and top strands). Modest enhancements of integration were seen at one edge of the protected region on both strands (around base 82 on the bottom strand and base 102 on the top strand). None of the other hot spots for integration seen with wt + zif complexes (Fig. 3 and 4) were seen with the purified zif DNA binding domain and wild-type preintegration complexes. Evidently combining wild-type preintegration complexes with free zif DNA binding domain is not sufficient to generate the hot spots seen in Fig. 3 and 4 with complexes containing the IN-zif fusion protein.

DISCUSSION

In this study, we present data indicating that (i) a new DNA binding domain could be introduced into infectious HIV-1 virions as a fusion with integrase and (ii) the new DNA binding domain altered integration site selection of preintegration complexes generated after infection. In initial studies, we found that integrase fusions were not well tolerated in virions (Fig. 2 and data not shown). However, IN-zif fusions were reasonably well tolerated as mixed multimers with wild-type integrase. Since there are probably many copies of integrase in each preintegration complex (unpublished data), complexes containing such mixtures will usually contain many copies of the zif268 domain. It is unclear whether the IN-zif monomers participated directly in catalysis or simply associated with preintegration complexes without participating in the covalent chemistry of integration.

Reactions with preintegration complexes containing the IN-zif fusion showed a distinctive pattern of integration site preferences in the presence of zif-binding sites. Integration hot spots appeared in the region of the zif sites. Weaker hot spots could be seen at more distant sites. Cold spots could also be seen at certain sites. Addition of free zif DNA binding domain to reactions with unmodified (wild-type only) complexes did not suffice to produce the hot spots observed with wild-type + zif complexes. Evidently, the hypothetical presence of free zif protein in the reaction mixtures could not account for the observed biased integration with wild-type + zif complexes.

During normal integration in vivo, the two 3′ ends of the viral cDNA are joined to protruding 5′ ends in the target DNA. These points of joining are offset on the two strands by 5 bp (4, 41, 42). Many of the hot spots for integration by the wild-type + zif complexes are separated by about 5 bp on the two target DNA strands (Fig. 4). Evidently, the higher-order architecture of wild-type preintegration complexes is sufficiently intact in wild-type + zif complexes for the expected 5-bp stagger to be preserved. The reason for the slight departures from the expected 5-bp spacing seen with some pairs of sites is unknown.

The pattern of hot spots and cold spots seen with zif-containing preintegration complexes differed from the pattern seen with the purified λR-IN fusion studied previously. For λR-IN, the integration hot spots clustered on the same side of the DNA helix as the repressor binding sites, while for the zif-containing complexes, there was no obvious favoring of one side of the helix. With the λR-IN protein, the repressor binding sites were protected from integration, while with the zif-containing complexes, hot spots were seen within the zif sites. The basis for these differences is at present unclear. The presence of two zif sites in the target DNA was functionally significant, since more pronounced selective integration was seen when two sites were present than when only one was present (data not shown), but it is not clear whether both sites were bound simultaneously during integration. For example, complexes bound at zif site 1 might not be bound at zif site 2 also and so might be able to carry out integration within site 2.

The inclusion of the zif domain in the IN-zif complexes altered integration site selection but did not remodel specificity completely. The formation of hot spots by zif-containing complexes indicates more frequent integration in the presence of zif sites, but substantial levels of integration were seen in target DNAs lacking zif sites altogether. For potential applications requiring higher levels of selectivity, it may be helpful to use recently reported derivatives of zif268 with increased affinity and specificity (43). In addition, since selectivity was improved in targets containing two zif sites instead of one, still greater selectivity may be seen with integration targets containing three or more sites.

The zif-containing preintegration complexes resemble several naturally occurring recombination systems. The Ty retrotransposons of yeast normally carry out highly selective integration with integrase enzymes similar in function and sequence to those of retroviruses. The Ty3 element, the most extreme case, integrates almost exclusively at the start site of transcription of genes transcribed by RNA polymerase III (10). Ty3 integration specificity has been proposed to be mediated by binding of the Ty3 integration apparatus to RNA polymerase III transcription factors (30). A tethering mechanism is also thought to mediate the local action of a variety of enzymes involved in DNA transposition and site-specific recombination. Many such enzymes are composed of modular domains for catalysis and site-specific DNA binding (2). The data presented here represent a step toward engineering such tethering systems into useful retroviral derivatives.

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