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Tethering human immunodeficiency virus 1 integrase to a DNA site directs integration to nearby sequences

(recombination/retrovirus/gene therapy/λ repressor/DNA binding)

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ABSTRACT **Certain retrovirus and retrotransposons display strong biases in the selection of host DNA sites for integration. To probe the possibility that simple tethering of the retroelement integrase protein to a target DNA site is sufficient to direct integration, the activities of a hybrid composed of human immunodeficiency virus 1 integrase and λ repressor were analyzed. In *in vitro* reactions containing several target DNAs, the λ repressor–integrase hybrid was found to direct integration selectively to targets containing λ operators. Addition of λ repressor blocked selective integration, indicating that binding to the operators was required. The λ repressor–integrase hybrid protein directed integration primarily to sites near the operators on the same face of the B-DNA helix, indicating that target DNA was probably captured by looping out the intervening sequences. Such hybrid integrase proteins may be useful for directing retroviral integration to specific sequences *in vivo*.**

The early steps of integration of retroviral DNA are carried out by the viral-encoded integrase (IN) protein acting on DNA sites at each end of the unintegrated viral DNA. Prior to integration, the IN protein cleaves the 3′ end of the blunt-ended product of reverse transcription to remove two bases. IN next joins the recessed 3′ ends of the viral DNA to 5′ ends of breaks made on each strand of the target DNA. The remaining unjoined strands are then connected, probably by host DNA repair enzymes, to yield an integrated provirus. Purified IN is able to carry out terminal cleavage and strand transfer reactions *in vitro* that model the formation of the integration intermediate described above (for a recent review, see ref. 1).

The integration of cDNA of retroviruses and retrotransposons can take place at many sites in the host genome, though the distribution of integration sites is not random. For Rous sarcoma virus, it has been reported that certain sites are used as targets a million times more frequently than expected (2). The mechanism of this targeting is unclear. Other departures from random integration site selection have been reported and in some cases attributed to effects of host proteins bound to the chromosomal target (3, 4). The Ty retrotransposons of *Saccharomyces cerevisiae* display extreme biases in integration site selection. Ty transposition involves reverse transcription and integration mediated by reverse transcriptase and IN enzymes similar in function and sequence to those of retroviruses (for review, see ref. 5). Of 32 unselected Ty1 integration sites generated in yeast chromosome III, not one was found to lie in a cellular gene, despite the fact that chromosome III is 70% coding region (6). Integration sites clustered upstream of tRNA genes and near preexisting Ty insertions. Ty3 displays an even more striking bias in target site selection. Efficient integration occurs only within 4 nt of the position of transcription initiation of tRNA genes. Binding of the Ty3 integration apparatus to polymerase III transcription factors has been proposed to direct integration to the start site of the tRNA gene (7). Possibly a similar interaction between Ty1 and a host chromosomal protein explains the biases in Ty1 integration.

Can simple tethering of a retroviral IN protein to a DNA site direct integration to local sequences? To investigate this issue, human immunodeficiency virus 1 (HIV-1) IN was fused to the DNA binding domain of λ repressor (λR), and target selection by the hybrid protein was compared with wild-type IN in assays *in vitro*. The λR–IN fusion was found to direct integration preferentially to targets containing λ operators. Wild-type HIV-1 IN, however, directed integration into DNA targets with similar frequency regardless of the presence or absence of λ operators. Evidently tethering IN to a target is sufficient to control integration site selection.

MATERIALS AND METHODS

DNA Manipulations. A plasmid encoding the N-terminal domain of λR fused to the leucine zipper of *GCN4* (pJH370) served as the cloning vector (8) for constructing the λR–IN coding region. To remove an interfering *Nde* I restriction enzyme recognition site from pJH370, a *Pst* I–*Bsa*AI fragment from pET15b (Novagen) was substituted for the corresponding fragment in pJH370, yielding pFB257 (9). This plasmid was then cleaved with *Nde* I and *Bam*HI and ligated with an *Nde* I–*Bam*HI fragment encoding HIV-1 IN as described (10), yielding pFB258.

Protein Purification. λR–IN was purified from cultures of induced *Escherichia coli* cells essentially as described for wild-type HIV IN (11) except that λR–IN was purified by ammonium sulfate back-extraction prior to column chromatography, and the butyl-Sepharose chromatography step was omitted.

Phage repressors were purified as described in ref. 12 and references therein.

Integration Assays. For integration assays in Figs. 1 and 2, an oligonucleotide of sequence 5′-ACTGCTAGAGATTTTCCACACGGATCCTAGGCTTTTGCCTAGGATCCGTGTGGAAAATCTCTAGCA-3′ (FB79) was used as substrate. This DNA can form a hairpin that resembles the U5 end of the HIV DNA after cleavage by IN to remove 2 nt. For the PCR assay, a duplex oligonucleotide resembling U5 was constructed by annealing 5′-ACTGCTAGAGATTTTCCACACGGATCCTAGGC-3′ (FB64) and 5′-GCCTAGGATCCGTGTGGAAAATCTCTAGCA-3′ (FB65-2). FB79 was labeled by treatment with [γ-^{32}P]ATP and T4 polynucleotide kinase.

Integration reactions were carried out as described (13) except that 2.5 μg of phage λ DNA (*cI857 indI Sam7*) was

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Abbreviations: HIV-1, human immunodeficiency virus 1; λR, λ repressor; IN, integrase; O_R and O_L, right and left operators, respectively.

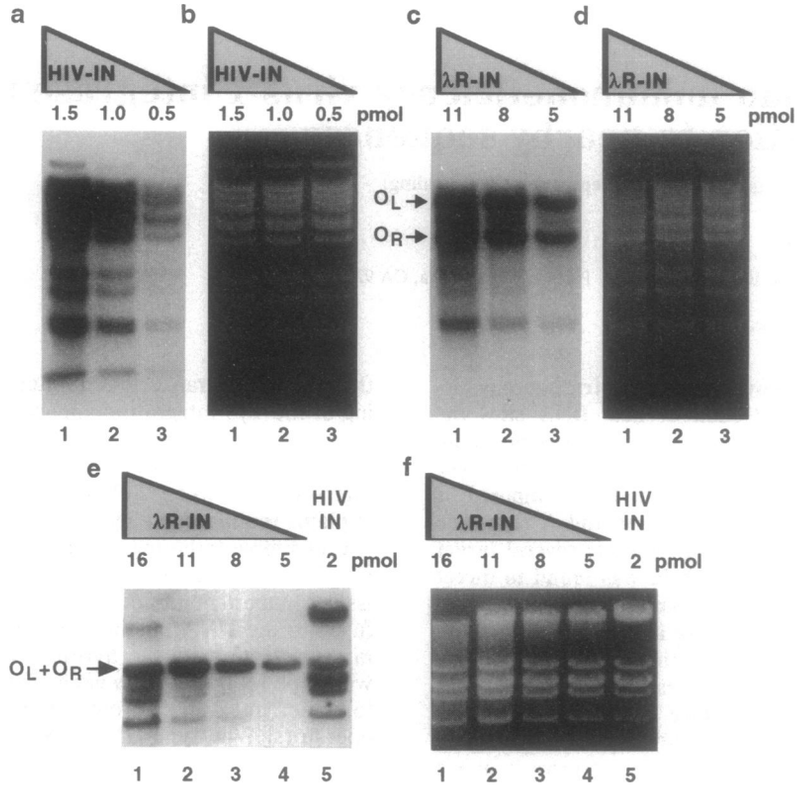

FIG. 1. Integration into λ DNA directed by λR–IN and HIV IN. (*a* and *b*) Products from reactions containing wild-type IN. (*c* and *d*) Products from reactions containing λR–IN generated using λ DNA cleaved with *Bst*EII as target. (*a* and *c*) Autoradiographic signals. (*b* and *d*) Photographs of gels stained with ethidium bromide. The amount of wild-type IN or λR–IN in each reaction mixture is marked above the lanes. The DNA fragments containing left operator (O_L) and right operator (O_R) are marked. (*e* and *f*) Products from integration reactions using λ DNA cleaved with *Eco*RI as target.

added as target. IN or λR–IN was preincubated with target on ice for 20 min, and reactions were started by adding the U5 donor DNA and transferring the reaction mixture to 37°C. After incubation for 20 min, reaction mixtures were heated at 60°C for 3 min (to melt the annealed λ DNA cohesive ends) and resolved on a Tris/Acetate/EDTA/0.7% agarose gel (9).

Integration reactions for analysis of products by PCR were carried out as above, except FB64/FB65-2 were used as the oligonucleotide donor. Reactions were stopped as above, deproteinized by phenol extraction, ethanol-precipitated, and resuspended in 20 μl of 10 mM Tris·HCl, pH 7.9/0.1 mM EDTA. Each PCR mixture contained one primer (FB66) complementary to the U5 donor oligonucleotide (sequence, 5′-GCCTAGGATCCGTGTGGAAAATC-3′) and a second primer complementary to λ DNA near the right operator, either FB182 (5′-GACAGATTCCTGGGATAAGCCAAG-3′, λ nt 37,830–37,853), or FB183 (5′-CGCGCTTTGATAGATATACGCCGAGATC-3′, λ nt 38,104–38,127). PCR mixtures contained 4 μl of integration product, 0.2 μg of each primer (FB66 and either FB182 or FB183), all four dNTPs (each at 0.25 mM), 1× *Taq* buffer (Stratagene), bovine serum albumin (0.1 mg/ml), and 2.5 units of *Taq* polymerase (Stratagene) in a final volume of 50 μl. Reactions were subjected to 30 cycles of amplification at 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Labeled products were analyzed on a denaturing 6% gel and visualized by autoradiography. Results were quantitated using a Molecular Dynamics PhosphorImager.

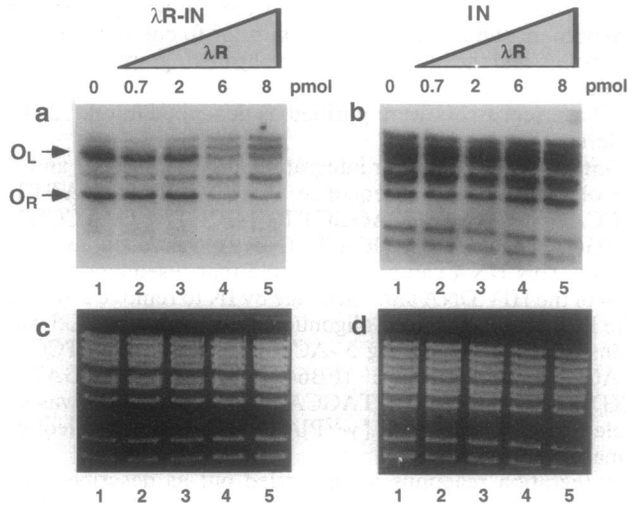

FIG. 2. Competition by phage repressors of specific integration directed by λR–IN. (*a* and *c*) Products of reactions containing λR–IN. (*b* and *d*) Products of reactions containing wild-type IN. The amount of λR added is indicated above the lanes.

RESULTS

Experimental Plan. The following considerations guided the design of the λR–IN fusion. λR is composed of two independently folded protein domains connected by a flexible linker. The N-terminal domain binds operator DNA, and the C-terminal domain mediates dimerization and tetramerization. λR binds as a dimer to a twofold symmetric DNA operator. Contacts between repressor and operator lie primarily on one face of the DNA helix, though a few contacts are also made to the back of the operator by a basic N-terminal arm that wraps around the DNA (12). The λR DNA binding domain and linker only (residues 1–133) were fused to IN in the hopes of minimizing the disruption of the active IN multimer by the added amino acids. The repressor

domain was fused to the N terminus rather than the C terminus of IN to preserve the flexibility of the repressor N-terminal arm and because IN remains functional despite the fusion of proteins to the N terminus (10, 14).

DNA Binding and Catalytic Activities of λR–IN. A genetic assay was used to monitor binding of λR–IN to λ operators. *E. coli* cells expressing λR–IN or control proteins were exposed to λ phage KH54. Cells expressing λR residues 1–133 only were lysed by this phage. Cells expressing λR–IN, however, survived, indicating that λR–IN is capable of binding to λ operators (data not shown). These observations also imply that the IN portion of the fusion directs dimerization of the λ DNA binding domains (8).

λR–IN was purified and tested for the known activities of HIV-1 IN. Prior to the integration of retroviral DNA, IN removed 2 nt from each 3′ end. Purified IN can carry out this terminal cleavage reaction *in vitro* on substrates that mimic one viral DNA end. Reactions containing λR–IN also yielded the expected terminal cleavage product. In addition, λR–IN was able to integrate the recessed 3′ end produced in this reaction into target DNA molecules present in the reaction mixture (see below).

Integration Site Selection by λR–IN. To probe integration site selection, reactions were carried out in which phage λ DNA was used as an integration target. λ DNA contains two groups of repressor binding sites, O_R and O_L. Target λ DNA was cleaved with *Bst*EII, yielding separate fragments containing O_L and O_R and several other fragments lacking operators that served as controls. Purified HIV IN or λR–IN was preincubated with 2.5 μg of *Bst*EII-cleaved λ DNA, and integration reactions were started by adding an end-labeled viral end oligonucleotide. After incubation for 30 min at 37°C, reactions were stopped and DNA products were analyzed by electrophoresis on native agarose gels. Integration products were visualized by autoradiography (Fig. 1 *a* and *c*) and total λ DNA was visualized by staining with ethidium bromide (Fig. 1 *b* and *d*). The unincorporated oligonucleotide viral end was not retained on the gel due to its short size. Because the viral end is much shorter than the λ target DNA fragments, integration does not result in a detectable change in the mobility of the integration target. The observation that the target DNAs in Fig. 1 retain the mobility of the unreacted target fragment implies that both wild-type IN and λR–IN are integrating primarily single viral end substrates into one strand of the target DNA as has been reported (13, 15).

Reactions containing wild-type IN yielded signals at each of the λ DNA fragments (Fig. 1*a*). Reducing the amount of IN reduced the signal but did not alter the relative signals of each fragment. Integration signals from reactions containing λR–IN, in contrast, were unevenly distributed, with integration favored in the fragments containing O_L and O_R (Fig. 1*c*). Reactions containing less of the λR–IN protein yielded less total integration product, but the O_L and O_R fragments contained an even greater proportion of the total integration signal (Fig. 1*c*, compare lanes 1 and 3). Eleven picomoles of λR–IN probably represents a functional excess of active multimers over operator sites, while 5 pmol fails to saturate the operator sites.

λR–IN also directed selective integration into target λ DNA cleaved with other restriction enzymes. Cleavage of λ DNA with *Eco*RI yields a single DNA fragment containing O_L and O_R. Products of integration reactions containing wild-type IN displayed similar autoradiographic signals at each λ DNA band (Fig. 1*e*, lane 5). Products of reactions containing λR–IN, in contrast, displayed stronger signals at the fragment containing O_L and O_R (Fig. 1*e*, lanes 1–4). The bias was again most pronounced at the lowest concentration of the λR–IN fusion (Fig. 1*d*, lane 4). Selective integration by λR–IN was also observed in reactions using λ DNA targets cleaved with other restriction enzymes (data not shown).

Competition of Site-Specific Integration by Phage Repressors. To challenge the idea that the λR–IN fusion directed selective integration by binding to λ operators, competition experiments were carried out by adding phage repressors. Fig. 2*a*, lane 1, shows the products of integration into λ DNA cleaved with *Bst*EII generated by 11 pmol of λR–IN. At this concentration, integration was favored in fragments containing O_L and O_R, but lower levels of integration into other fragments were also detected. Addition of 0.7–2 pmol of λR had little effect (Fig. 2 *a* and *c*, lanes 2–4). Upon addition of 6 pmol of λR, however, the pattern of site selection was changed such that integration events were evenly distributed on the target DNA (Fig. 2*a*, lane 5). For reaction mixtures containing 2 pmol of wild-type IN, integration sites were evenly distributed over all the DNA fragments, and addition of repressor did not change this pattern (Fig. 2 *b* and *d*).

As a control, the experiment was repeated using the repressor protein of the heteroimmune lambdoid phage 434 as competitor. Phage 434 repressor acts at the 434 right operator in a manner closely similar to that described for λR (12, 16), but 434 repressor binds specifically only to 434 operators (12). Titration of 434 repressor into integration reactions did not influence the selection of integration target, supporting the view that λR–IN directed selective integration by binding to λ operators.

High-Resolution Analysis of Integration Sites in O_R. A PCR-based assay (4, 17) was used to determine the efficiency of integration at each phosphodiester in the vicinity of O_R. Integration reactions were conducted essentially as above except that the viral end oligonucleotide was not end-labeled. Products of integration reactions were deproteinized and used as templates for PCR. PCR primers were selected so that one primer was complementary to a target DNA sequence, and the other was complementary to the viral end oligonucleotide. The target primer was labeled with ^{32}P on the 5′ end. PCR amplification of integration products generated a population of molecules that were denatured and analyzed on a DNA sequencing type gel. Each band on the gel corresponded to integration at a specific phosphodiester. The frequency of integration at a particular site was reflected in the intensity of the band. The location of each integration site was determined by coelectrophoresis of DNA sequencing reactions with PCR products. PCR primers to either side of O_R were used to examine integration into each DNA strand.

Such a PCR analysis of sites used by HIV IN in the λ DNA target is presented in Fig. 3 *a*, lane 1 (top strand), and *b*, lane 1 (bottom strand). A band can be seen at most positions in the product ladder, indicating that most phosphodiesters in the λ DNA can serve as target. Relative efficiencies of integration, however, vary over at least a 100-fold range. The effect of added λR on site selection was also determined (Fig. 3 *a* and *b*, lanes 2–6), in part to facilitate the interpretation of the pattern seen with λR–IN described below. In the presence of 6 pmol of λR, the region containing O_R1 and O_R2 was fully protected, presumably because λR at these sites obstructed access of IN. Higher levels of λR, 18 pmol or more, were required to fill the low-affinity site, O_R3 (Fig. 3 *a* and *b*, lanes 5 and 6). These data further support a previous study (4) that indicated that integration followed by PCR can be used to detect DNA-bound proteins as in established "footprinting" protocols.

The presence of bound λR enhanced integration at several sites adjacent to the operators. This effect was particularly prominent at two positions on the top strand (Fig. 3*a*; PCR product sizes, 206 and 226 nt). The enhancement at nt 206 is maximal as O_R1 and O_R2 fill (2 pmol of λR) but was suppressed as λR fills O_R3 (54 pmol of λR). Evidently the presence of λR at O_R2 stimulates integration at an adjacent site in O_R3, but filling of O_R3 blocks integration into this site. The site at nt 226, in contrast, was prominent only at high

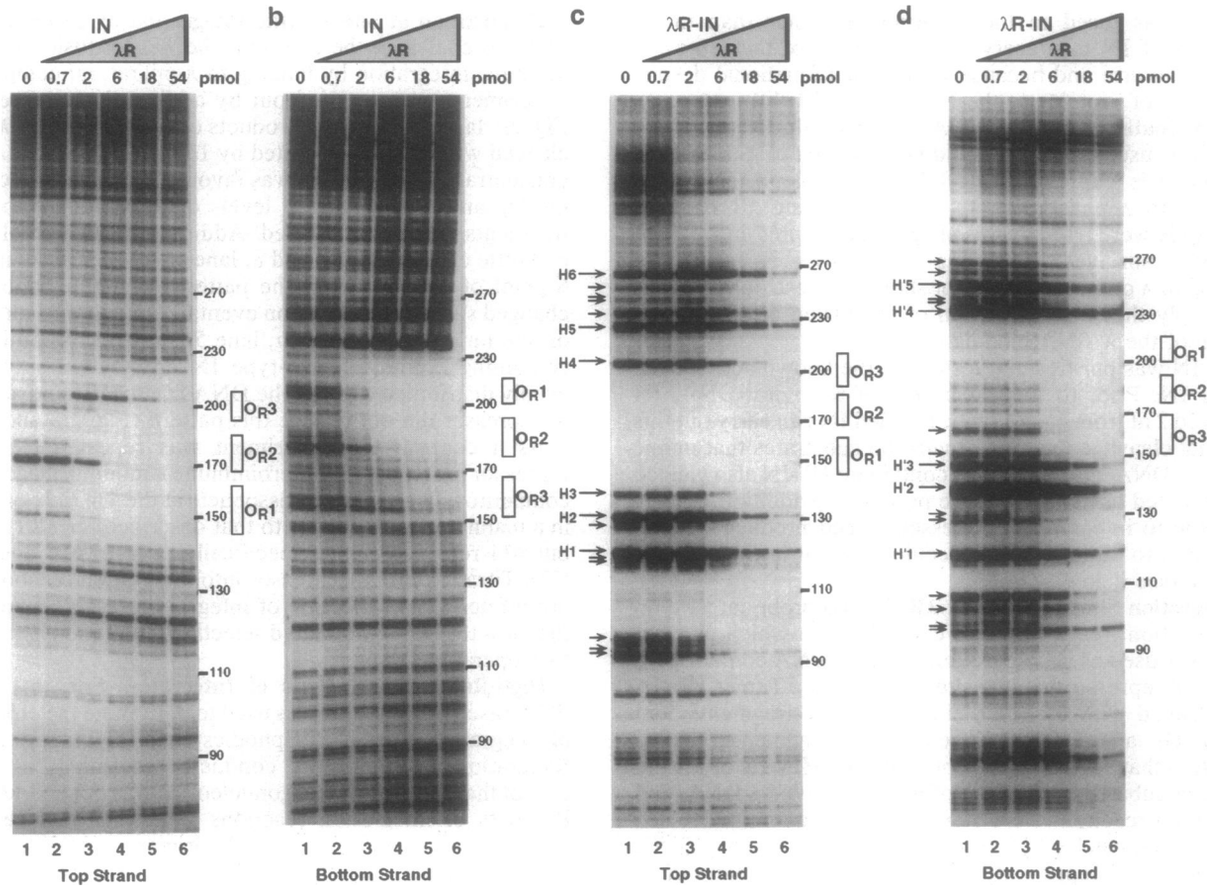

FIG. 3. High-resolution mapping of integration sites used in reactions containing wild-type IN or λR–IN and the effects of added λR. Integration sites used by wild-type IN on the top strand (*a*) and on the bottom strand (*b*) of the target DNA and analysis of integration sites used by λR–IN on the top strand (*c*) and on the bottom strand (*d*) are shown. Reaction mixtures contained 2 pmol of wild-type IN or 5 pmol of λR–IN. The amount of λR added to each integration reaction is indicated at the top. The sizes of the amplification products, determined by coelectrophoresis with DNA sequencing reactions, is shown to the right of each panel. The mobilities of fragments generated by integration into O_R1, O_R2, and O_R3 are as marked. To align the PCR products with the λ DNA sequence (compiled in ref. 18), 30 bases, the length of the integrated oligonucleotide substrate, was subtracted from the measured length of the PCR product to determine the distance of the integration sites from the location of the λ primer.

concentrations of λR (54 pmol). Use of this site appears to be stimulated by the presence of λR at O_R3.

A similar PCR analysis of integration sites used by λR–IN yielded a strikingly different pattern, dominated by strong enhancements to either side of O_R1 and O_R2 (Fig. 3 *c* and *d*). These sites on the top strand are labeled H (for hot spot) and numbered from 5′ to 3′. Bottom-strand hot spots are labeled H′. Each is marked with a long arrow. Less-prominent hot spots are marked by short arrows. λR–IN directed little integration into the O_R1 and O_R2 sites themselves, as expected if λR–IN occupied these sites. Integration was more prominent in O_R3, indicating that it is unoccupied or only partially occupied. Titrating λR into reactions containing λR–IN diminished integration into the hot spots near O_R but had less influence on the weak integration seen at more distant sites (Fig. 3 *c* and *d*, lanes 2–6, and data not shown). These data are as expected if integration at the hot spots near O_R is carried out by the λR–IN fusion protein bound at O_R.

Alignment of the periodic pattern on a DNA model revealed that many of these sites lie on one face of the DNA helix (Fig. 4). λR–IN is also expected to be bound to this side of the helix. These data are consistent with a model in which the λR–IN complex bound at O_R captures target DNA by looping out the intervening sequences.

DISCUSSION

In this study, a fusion protein containing the DNA binding domain of λR linked to the IN of HIV-1 (λR–IN) was found to direct integration preferentially into DNA containing λ operators. Wild-type HIV-1 IN, in contrast, showed no preference for DNA targets containing λ operators. Selective integration by λR–IN could be completed by addition of λR, indicating that operator binding was required. These findings strengthen the view that the observed selective integration by several IN proteins *in vivo* may be due to tethering the

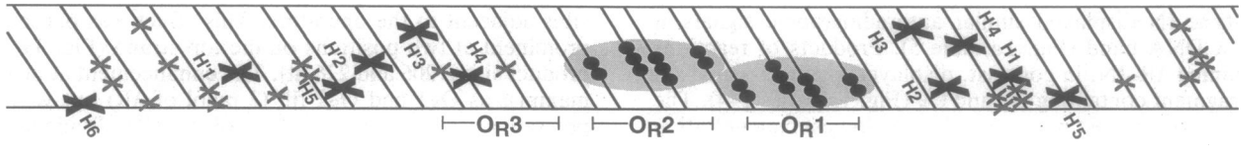

FIG. 4. Hot spots for integration by λR–IN (crosses) displayed on a projection of the B-DNA helix. Phosphates contacted by the repressor DNA binding domain at O_R1 and O_R2 (19) are marked with solid dots. The B-DNA helical repeat was modeled as 10.5 bp per turn.

recombination machinery to target DNA. Such fusions of IN to other proteins may serve as tools for controlling the site of retroviral DNA integration *in vivo* after infection.

Organization and Function of the λR–IN Complex at O_R. λR binds DNA as a dimer, and HIV-1 IN also acts as a dimer or higher-order multimer. The observation that λR–IN is capable of targeting integration to DNA near λ operators implies that the fusion protein multimerizes with the geometry necessary to allow function of both components. The operator-binding multimer of λR–IN probably itself forms a stable dimer, as indicated by the site occupancy in integration reactions. O_R1 and O_R2 are not used as target by λR–IN, indicating that λR–IN binds O_R1 and O_R2. O_R3 is used as an integration target by λR–IN, indicating that it is not efficiently occupied. The intrinsic affinities of O_R2 and O_R3 for repressor, however, are similar (12). Thus the efficient filling of O_R2 by λR–IN is probably due to a cooperative interaction with λR–IN bound at the higher affinity site O_R1. Normally λR itself fills O_R1 and O_R2 cooperatively, but this requires interactions between C-terminal domains, which are missing in the λR–IN hybrid. Thus the IN part of the fusion probably mediates higher-order multimerization of the λR DNA binding domains. As is required by this view, HIV-1 IN is known to be able to form tetramers (20). The actual λR–IN complex at O_R may be even larger than a tetramer. Purified IN is known to aggregate into large multimers under the conditions of assays *in vitro*, and HIV IN *in vivo* acts as a multimer probably composed of at least a hundred monomers (M. Miller and F.D.B., unpublished data).

Many of the hot spots for integration by λR–IN are separated by ≈10 bp on each strand, and the patterns on the two strands are offset by ≈5 bp. Such a pattern is characteristic of interactions on one face of the helix in normal B-DNA. The helical model in Fig. 4 displays integration hot spots and the positions of phosphates in close contact with the λR DNA binding domain bound at O_R1 and O_R2. The λR phosphate contacts lie on the same face of the helix as the periodic hot spots, consistent with a model in which λR–IN captures target by looping out the intervening DNA. Presumably the tortional rigidity of DNA reduces integration into the opposite side of the helix.

Several departures from the periodic pattern of hot spots may be due to effects of local sequences. Several turns of the DNA helix lack expected hot spots, perhaps because the local DNA sequences are unfavorable for integration. In support of this view, most positions in the periodic integration pattern missing expected hot spots are also cold spots for integration with wild-type IN. Similarly, the most prominent hot spots for integration by λR–IN are also relatively active target sites for integration by wild-type IN.

Two strong hot spots near O_R1 and O_R2, H3 and H′3, do not fit the periodic pattern. These sites do correspond, however, to sites that are enhanced in the presence of λR in reactions containing wild-type IN. Previous work has established that DNA distortion can promote integration (21), so perhaps distortion in the DNA emerging from the λR–operator complex at these positions promotes integration. However, little DNA distortion is seen in the x-ray structure of the λR DNA binding domain bound to an operator (22). Alternatively, the presence of λR near the favored sites may neutralize some of the negative charges on the DNA phosphates, thereby reducing the energy required to bring together the viral DNA end and the target DNA.

Possible Applications of Sequence-Specific Integration Systems. Retroviral derivatives containing fusions of IN to sequence-specific DNA binding domains may find uses in gene therapy. Despite the known biases in the selection of integration targets by retroviruses, it is clear that most chromosomal regions contain many potential integration sites. This presents a concern in the use of retroviruses as vectors for gene therapy, since retroviral integration can disrupt the target genome by insertional mutagenesis. Numerous studies in vertebrate model systems have established that integration of retroviral DNA can result in inactivation or ectopic activation of cellular genes, thereby causing diseases (23). The same safety concerns apply to the use of attenuated retroviruses as vaccines. The use of fusions between IN and site-specific DNA binding domains might permit integration to be directed to innocuous sites, thereby circumventing these potential problems. Retroviral vectors capable of sequence-specific integration might also find uses as insertional mutagens to disrupt destructive DNA sequences such as activated oncogenes or viral genomes.

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