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Integration of human immunodeficiency virus DNA: Adduct interference analysis of required DNA sites

(retrovirus/long terminal repeat/integration protein/acquired immunodeficiency syndrome/recombination)

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ABSTRACT **The integration (IN) protein encoded by human immunodeficiency virus directs the integration of viral DNA into host DNA. We have probed the DNA sites required for the function of IN protein by attaching adducts to model DNA substrates and assaying their effects on integration *in vitro*. These experiments reveal that modifications in a short region on both DNA strands at the ends of the viral DNA block IN protein function. Modification of the target DNA near the point of DNA strand transfer also blocks IN protein function. Further experiments suggest that distinct subsets of the identified interactions are important for separate steps in the integration process.**

Integration of retroviral DNA into host DNA involves a coordinated series of DNA cleavage and joining steps. The substrate for the integration system is the linear double-stranded form of the viral DNA made by reverse transcription of the viral RNA genome (1–5). Prior to integration, the flush ends of the linear viral DNA are cleaved so as to remove 2 bases from each 3′ end (1, 2, 6–11). These recessed 3′ ends are then joined to 5′ ends of breaks made in the target DNA (1, 2). According to our present understanding, host DNA repair systems process and ligate the remaining unjoined DNA ends in the integration intermediate, completing proviral synthesis. Selection of the target site is nonspecific with respect to DNA sequence (for reviews, see refs. 12 and 13).

The virus-encoded IN protein is required for integration of viral DNA *in vivo* (14–18) and is sufficient to carry out the synthesis of the integration intermediate *in vitro*. These *in vitro* integration systems require only purified IN protein, a reaction mixture containing $MnCl_2$, and model DNA substrates, which can be supplied as short duplex oligonucleotides that match the termini of the unintegrated viral DNA. Reaction products include (*i*) specifically cleaved DNAs in which 2 bases have been removed from the 3′ end that matches the viral DNA terminus (termed the "viral DNA end" in this report) (19–22) and (*ii*) strand transfer products in which the recessed 3′ termini of the cleaved viral DNA ends have been joined to target DNAs (see Fig. 1*A*) (5, 21–23).

Attempts to map interactions between the IN protein of human immunodeficiency virus (HIV) and DNA have been complicated by the lack of detectable specific binding in gel retardation and DNA "footprinting" experiments (24, 25) (A. Engelman and R.C., unpublished data). In addition, competitor challenge experiments of integration reactions *in vitro* revealed no DNA sequence specificity in the initial binding of HIV IN protein to DNA (24, 25) (A. Engelman and R.C., unpublished data). To determine the locations of functionally critical interactions between the HIV IN protein and DNA in the presence of this high background of nonspecific binding, we devised a method that relies on analyzing integration products rather than physically assaying binding.

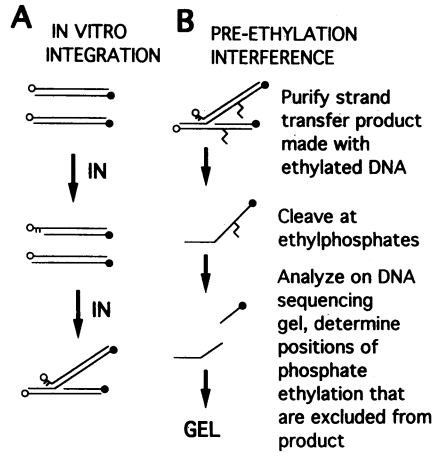

FIG. 1. Schematic diagrams of the *in vitro* integration assay (*A*) and the ethylation interference experiment conducted on the joined DNA strand (*B*). (*A*) Duplex oligonucleotide DNAs mimicking the U5 DNA end, shown here with label on the strand expected to be joined to target DNA (*Top*). DNA 5′ ends are shown as circles and labeled 5′ ends are shown as filled circles. In the presence of the HIV IN protein, the terminus of the model DNA substrate matching a viral DNA end can be cleaved so as to remove 2 bases from the 3′ end (*Middle*), and the recessed 3′ end can be joined to a 5′ end of a break made in the target DNA (*Bottom*). (*B*) In the ethylation interference experiment, strand transfer products were generated by IN protein as above with ethylated DNA molecules (*Top*). Ethyl groups are shown as a zigzag line. The DNA strand covalently joined to target DNA was isolated (*Middle*) and then cleaved at the positions of ethylphosphates by heating in NaOH (*Bottom*). Positions at which ethylation prevents DNAs from participating in the reaction are revealed as gaps in the ladder of NaOH cleavage products after electrophoresis and autoradiography.

MATERIALS AND METHODS

Ethylation Interference. End-labeled duplex substrate DNAs [long terminal repeat (LTR) J (22) or LTR K] were treated with ethylnitrosourea (Sigma) for 1 hr and purified as described (26). Two to five 80-μl integration reactions were performed with each ethylated DNA. Each reaction mixture contained 30 mM Mes (pH 6.5), 10% (vol/vol) glycerol, 7.5 mM $MnCl_2$, 10 mM 2-mercaptoethanol, 30 pmol of HIV IN protein, 1.5 pmol of ethylated oligonucleotide, 8 μg of bovine serum albumin, and 65 mM NaCl. Reaction mixtures containing DNAs labeled in the unjoined strand also contained 1 μg of phage φX174 replicative form (RF) DNA. Reaction mixtures were incubated for 2 hr at 30°C. Reaction products with label in the joined strand were isolated after electro-

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Abbreviations: HIV, human immunodeficiency virus; LTR, long terminal repeat.

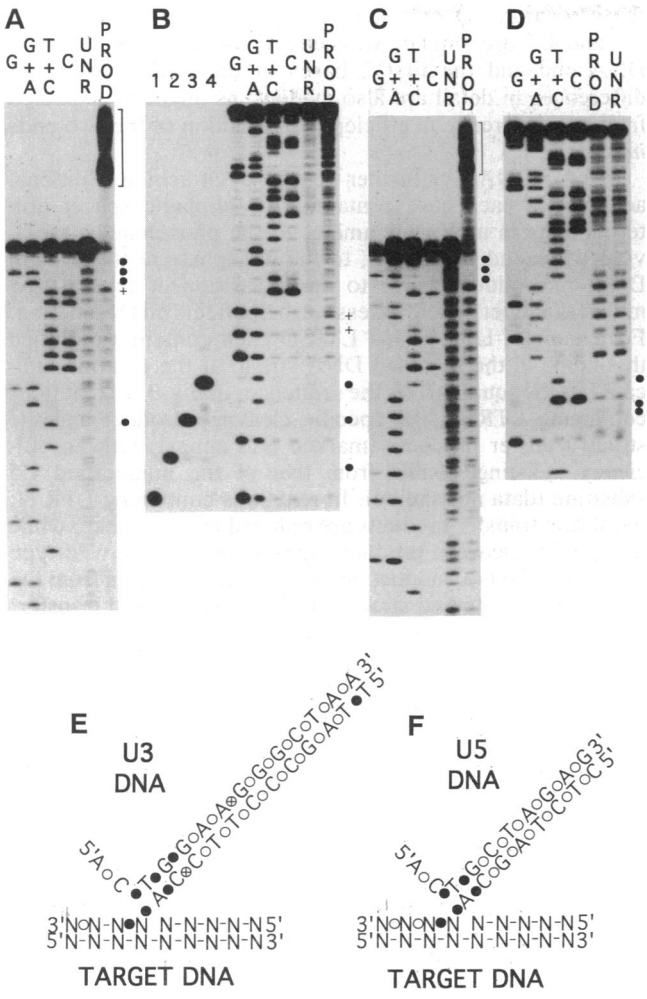

FIG. 2. Identification of positions at the HIV DNA termini where ethylation blocks integration *in vitro*. *A* and *B* present results with substrates matching the joined and unjoined strands of the U3 viral DNA end, respectively; *C* and *D* present results with substrates matching the joined and unjoined strands of the U5 viral DNA end. Lanes marked "UNR" contain control products of NaOH cleavage of unreacted ethylated DNA; lanes marked "PROD" contain products of NaOH cleavage of DNA strand transfer products made with ethylated DNA. Bands that are less abundant in the PROD lane than in the control UNR lane identify positions (●) where ethylation diminishes product formation. Ethylation at some positions (+) enhances product formation. Brackets in *A* and *C* indicate strand transfer products that were not cleaved by NaOH. Products of Maxam–Gilbert cleavage reactions performed on the unethylated substrate are shown in lanes G, G+A, T+C, and C. In *B*, lanes 1–4 contain mobility controls: [5′-^{32}P]dAMP (lane 1), d([5′-^{32}P]pApC) (lane 2), d([5′-^{32}P]pApCpT) (lane 3), and d([5′-^{32}P]pApCpTpG) (lane 4). In *A* and *C*, data for 20 base pairs (bp) at each viral DNA end are shown. *E* and *F* summarize the positions of ethylation interference and enhancement on strand transfer products made with U3 and U5 DNA, respectively. Phosphates assayed by ethylation interference are shown as circles. Filled circles indicate positions of ethylation interference, and circled plus signs indicate positions of ethylation enhancement. Phosphates that were not assayed are shown as dashes. Only the DNA regions containing ethylation effects are shown. The bands in *A–D* were correlated with the positions in the DNA sequence as follows. Cleavage by NaOH at positions of ethylphosphates generates hydroxyl or ethylphosphate 3′ termini (28). In longer DNA chains these products typically migrate just slightly more slowly than the corresponding Maxam–Gilbert cleavage product, which terminates with a 3′ phosphate (29). Thus the Maxam–Gilbert cleavage ladder could be used to assign band positions in *A* and *C*. This strategy could not be used for the cleavage products shown in *B* and *D*, because of their very short length: for very short DNA chains a difference of one phosphate can result in very large mobility shifts relative to Maxam–Gilbert standards (30, 31). Size markers were therefore synthesized corresponding to the expected 3′-hydroxyl-containing products of cleavage at ethylphosphates, and positions of cleavage were assigned by alignment with these standards (*B*, lanes 1–4; data not shown). The ethylphosphate-terminated cleavage products were not identified in *B* and *D*, because of the absence of a suitable marker. Further details of the interpretation are as follows. The band in *A*, lane PROD, at the mobility of the unreacted oligonucleotide could be due to contamination of the purified strand transfer product with unreacted oligonucleotide and so is indicated as not tested in *E*. The identity of the longer molecules in *B* and *D* for which we do not have matching mobility controls was tentatively assigned by counting up the "rungs" of the cleavage ladder. There is an ambiguity in assigning the two enhanced bands in *B*, which could be interpreted as two adjacent 3′-hydroxyl forms or one 3′-hydroxyl form and one 3′-ethylphosphate form. We chose the latter interpretation. The bottom pair of interferences in *D* are interpreted as 3′-hydroxyl and 3′-ethylphosphate forms derived from cleavage at a single ethylphosphate position, though we only have a mobility standard for the 3′-hydroxyl form. Only positions of strong ethylation effects are interpreted. Weaker effects that were not interpreted may nevertheless be of biological significance.

phoresis in a DNA sequencing gel (21). Reactions containing label in the unjoined strand were stopped by adding 5 μl of 0.5 M EDTA and 5 μl of 0.5% Pronase in 10 mM Tris, pH 8/0.5 mM EDTA and incubated for 2 hr at 37°C. Reaction mixtures were pooled and spun through Sephacryl S300 columns (roughly 800 μl of matrix per column, 200 μl of sample per column) in a Sorvall RC3B centrifuge (H6000A rotor) at 1000 rpm for 5 min. The excluded peak was then applied to a Waters GenPac HPLC column in 10 mM Tris, pH 8/0.5 mM EDTA and eluted with a linear gradient of 0.4–0.75 M NaCl in the same buffer. Product DNAs were concentrated by ethanol precipitation and cleaved at ethylphosphates by heating for 30 min at 90°C in 0.14 M (reactions in Fig. 2 *A*, *C*, and *D*) or 0.6 M (reaction in Fig. 2*B*) NaOH. Reducing the concentration of NaOH from 0.6 M (26) to 0.14 M reduced the accumulation of products that comigrated exactly with the Maxam–Gilbert chemical cleavage ladder. The electrophoretic mobility markers in Fig. 2*B* were synthesized by (*i*) treatment of [α-^{32}P]ATP with snake venom phosphodiesterase or by treatment of (*ii*) d(ApC) (Pharmacia) or synthetic oligonucleotides (*iii*) d(ApCpT) and (*iv*) d(ApCpTpG) with [γ-^{32}P]ATP and polynucleotide kinase.

Primary Amine Interference. Adducts were incorporated into oligonucleotides as described (27): adduct 1, 3-amino-2-hydroxy-propyl 5′ phosphate ester; adduct 2, hexylamine 5′ phosphate ester; and adduct 3, 5-[(6-aminohexyl)-3-acrylimido]-2′-deoxyuridine. The presence of each adduct was confirmed by establishing that each modified DNA could be efficiently biotinylated, as assayed by a reduction in mobility upon electrophoresis in a DNA sequencing gel, or by binding to avidin-coated beads, or by both. The efficiency of biotinylation was typically about 80%.

HIV DNA Integration *in Vitro*. Integration reactions were conducted as described (22), except that 2 pmol of IN protein, overexpressed in *Escherichia coli* (20) (A. Engelman and R.C., unpublished work) and purified to near homogeneity essentially as described (20), was used in each 16-μl reaction. Labeled DNAs were visualized by autoradiography after electrophoresis in a 20% polyacrylamide DNA sequencing gel. Results were quantitated with a Molecular Dynamics (Sunnyvale, CA) PhosphorImager.

RESULTS

To determine the locations of functionally important interactions between IN protein and DNA, duplex substrate oligonucleotides were modified by attaching adducts to various positions on the DNA, and the positions at which

adducts interfered with product formation were identified. In one set of experiments, 5′-labeled oligonucleotides matching either the U3 or the U5 terminus of the unintegrated viral DNA were ethylated by treatment with ethylnitrosourea, which attaches ethyl groups to the phosphate backbone (26). Each DNA strand contained on average less than one ethylphosphate. Ethylated DNA molecules were next incubated with IN protein under reaction conditions. Strand transfer products were separated from unreacted substrate DNA, purified, and then cleaved at the positions of ethylphosphates by heating in NaOH. Cleavage at each position of phosphate ethylation yields a labeled DNA molecule of a specific length. After electrophoresis in a DNA sequencing gel, molecules of each length form a distinct band. Cleavage of a population of randomly ethylated end-labeled molecules yields a regular "ladder" of cleavage products. Cleavage of integration products made with ethylated DNA also yields a regular ladder, except that gaps are seen in the ladder at positions where ethylation prevents DNAs from participating in the reaction.

Separate experiments were performed to analyze interactions with each DNA strand. A ^{32}P label was attached either to the 5′ end of the substrate strand that becomes covalently joined to target DNA (diagramed in Fig. 1) or to the 5′ end of the unjoined DNA strand. In the first case, no special target DNA was added to the reaction, and ethylated oligonucleotides served as both viral DNA ends and target (Fig. 1). Strand transfer products were isolated after denaturation as labeled DNAs of higher molecular weight than the unreacted oligonucleotide (Fig. 1*B Middle*) and analyzed by electrophoresis after cleavage in NaOH (Fig. 1*B Bottom*). Interactions with a part of the target DNA could also be analyzed in this experiment, because the ethylated oligonucleotide DNA also served as target. For the case of strand transfer products made with the 5′ label in the unjoined strand, phage ϕX174 RF DNA was used as an integration target, and products were isolated without denaturation.

Experiments were performed with model substrates matching the U3 (Fig. 2 *A* and *B*) and U5 (Fig. 2 *C* and *D*) ends of the unintegrated HIV DNA. The intensity of each band in the control lane (UNR), which contains NaOH cleavage products of the unreacted ethylated oligonucleotide, shows the intrinsic efficiency of ethylation at each phosphate. Bands resulting from NaOH cleavage that are of lesser intensity in the strand transfer products (PROD) relative to the control lane (UNR) mark phosphates at which ethylation diminishes the participation of that DNA molecule in the reaction. These positions are indicated by filled circles beside the autoradiograms in Fig. 2 *A–D*. To facilitate identification of specific products, some of the expected products were synthesized and subjected to electrophoresis in adjacent lanes (Fig. 2*B*, lanes 1–4). Two positions at the U3 DNA end where ethylation *enhances* reactivity are marked by the plus signs in Fig. 2 *A*, *B*, and *E*.

Positions of ethylation interference cluster on both U3 and U5 at the viral DNA end near the phosphates where cleavage and joining take place [filled circles in Fig. 2 *E* (U3) and *F* (U5)]. Positions of inhibitory ethylations are found on both DNA strands. Two more inhibitory ethylphosphates are found in the target DNA on the joined strand, one at the junction between viral DNA and target DNA, and the other at the adjacent target phosphate. At the U3 end only, an additional sensitive phosphate is found on the joined strand about one helical turn away from the cluster of interactions near the viral DNA ends. Ethylation at other positions in this region may also show inhibitory effects. The U3 ethylation enhancements probably mark positions at which an ethyl group interacts with IN protein so as to facilitate product formation, or else the neutralization of the phosphate negative charge that accompanies ethylation promotes IN function. In either case, IN protein probably closely approaches these positions. Overall, the patterns of ethylation effects at U3 and U5 are similar, with effects clustering at the viral DNA end and the first 2 bases in the target DNA, but differences in detail are also evident, as might be expected from the difference in efficiency of function of the two ends *in vitro* (22).

The U5 DNA was further probed by attaching additional adducts, in each case containing an aliphatic spacer arm terminating in a primary amine, to the phosphate at the 3′ viral DNA end (adduct 1), to the phosphate at the 5′ viral DNA end (adduct 2), or to the 5 position of several thymidines (adduct 3), and assessing their effects on IN function. For example, LTR N and LTR O each contain a modified thymidine in the unjoined DNA strand at the position indicated by the squares over the sequences in Fig. 3. In reactions containing LTR O, the specific cleavage product and the strand transfer products (marked ST) appear with an efficiency indistinguishable from that of the unmodified U5 substrate (data not shown). In reactions containing LTR N, the strand transfer products are reduced in abundance, while the specific cleavage product appears with at least wild-type efficiency. Thus an adduct on the thymidine 3 bases from the 5′ end of the unjoined strand interferes with strand transfer; the intriguing separation of specific cleavage and strand transfer activities is discussed below.

In further experiments, the primary amines in the adducts on LTR N and LTR O were biotinylated, thereby attaching a 19-atom chain terminating with a biotin moiety to the DNA, and the activities of the biotinylated DNAs were assayed as above. Reactions containing LTR N–biotin showed a diminution of strand transfer comparable to that seen with LTR N, whereas reactions containing LTR O-biotin showed some inhibition of strand transfer (data not shown). Lengthening

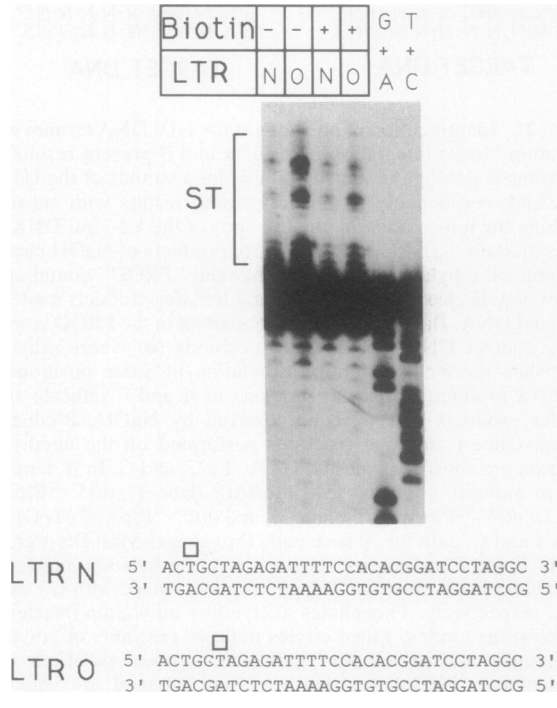

FIG. 3. Specific cleavage and strand transfer products obtained in reactions containing HIV IN protein and LTR N and LTR O. LTR N and LTR O are identical to the U5 substrate except that each is modified by addition of a primary amine at the thymidine indicated by the square over the sequence. Lanes marked + contain the indicated LTR substrate modified by biotinylation. Lanes marked G+A and T+C contain the products of Maxam–Gilbert chemical cleavage reactions. Position of DNA strand transfer products is marked (ST). LTR N and LTR O were labeled with ^{32}P at the 5′ end of the bottom DNA strand.

FIG. 4. Summary of results of adduct interference experiments carried out on the U5 DNA end. The sequence of the U5 DNA end is as shown; the viral DNA end is to the left. Filled symbols, positions where adducts diminish the accumulation of strand transfer products at least 3-fold; open symbols, positions where adducts do not interfere with function; oval, primary amine at the terminal phosphate; square, primary amine at thymidine methyl; diamond, biotinylated side chain at the 5 position of thymidine. Biotinylation of adduct 3 (at the thymidine methyl) yielded 5-[*N*-(biotin-XX-aminohexyl)-3-acrylimido]-2'-deoxyuridine (X = 6-aminohexanoyl).

the DNA adduct at LTR O may permit the adduct to encounter IN protein in a way that diminishes strand transfer. Fig. 4 summarizes DNA adduct interference results on the U5 DNA.

Adducts attached to 5' or 3' phosphates at the viral DNA end do not block IN function, and the viral DNA end can be lengthened or shortened by a base pair (22, 24), suggesting that IN protein does not interact intimately with the terminal structure at the viral DNA end. To determine whether a nearby DNA end is in fact important for function, as has been suggested for other retroviral integration systems (6, 32, 33), we synthesized and analyzed a model substrate in which 14 bp of DNA was attached to the viral end of a 21-bp HIV U5 substrate. No specific cleavage or strand transfer products were detected (data not shown), indicating that a nearby DNA end is required for the function of HIV IN protein.

The inhibition of strand transfer seen upon ethylation of critical phosphates could have occurred at any step in the integration process prior to the completion of strand transfer. An experiment that begins to dissect the contributions of specific interactions to each of the steps in DNA integration is shown in Fig. 5. LTR K, a 32-bp U5 oligonucleotide, serves as a positive control. In the presence of IN protein (lane +) the specific cleavage (−2) and strand transfer (ST) products are produced. However, if two nucleotides are removed from the 5' end of the unjoined strand (LTR L), specific cleavage is enhanced about 3-fold whereas strand transfer is inhibited. Strand transfer is also diminished in a substrate missing the terminal 2 bases on both strands (data not shown). LTR N, which contains a primary amine attached to the thymidine 3 bp from the 5' end of the unjoined strand, also shows a suppression of strand transfer and a possible increase in specific cleavage. These results indicate that some interactions on the unjoined strand are critical for the strand transfer step but dispensable for the specific cleavage step of integration.

DISCUSSION

In this study, we map sites at the termini of the unintegrated HIV DNA that are critical for DNA integration by attaching adducts to model DNA substrates and assessing their effects on integration *in vitro*. Most of the positions of adduct interference lie in a small cluster near the ends of the terminal HIV sequences; for the case of the U3 end, two further positions of adduct effects lie at more distal positions. Positions of inhibitory ethylations also lie in the target DNA at the two phosphates nearest the point of strand transfer on the joined DNA strand. Studies of mutant DNA substrates indicate that only a few base pairs at the viral DNA termini, particularly the 5'-CA-3' sequence at the point of strand transfer, are critical for integration *in vitro* (22, 24, 25) or replication of HIV *in vivo* (E. Vicenzi and M. Martin, personal communication), and most of the positions of strongest adduct interference lie near the 5'-CA-3' sequence.

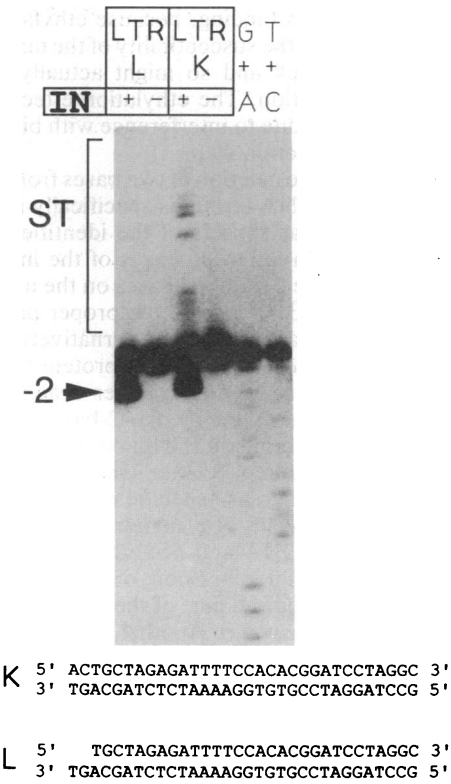

FIG. 5. Specific cleavage and strand transfer products obtained in reactions containing HIV IN protein and U5 (LTR K) or deleted U5 (LTR L) model substrates. Labeling of the autoradiogram is the same as in Fig. 3. DNA substrates were labeled with ^{32}P at the 5' end of the bottom DNA strand.

In vivo, HIV IN protein acts as a part of a stable high molecular weight complex derived from the viral core (3, 4). Since interactions within the nucleoprotein complex may help to hold IN near its site of action, a relatively small number of interactions may be sufficient for IN protein function.

How can the finding that specific DNA sites at the viral DNA end are required for function be reconciled with the observation that IN protein binds nonspecifically in DNA-binding competition experiments? In principle, the specificity revealed in the interference experiments can be required at any reaction step, and specificity may not be detected in binding studies because it is required only for the later chemical steps of the reaction. Such a model has been proposed for sequence-specific cleavage by the restriction endonuclease *Eco*RV, which binds equally tightly to specific and nonspecific DNA sites but cleaves only at its recognition site (34). In the HIV IN case, specificity could also be required at the initial binding step to form a productive complex but not be detected in binding experiments because sequence-nonspecific complexes dominate the population of complexes formed *in vitro*.

DNA adducts have been found to block the binding of many site-specific DNA-binding proteins. Ethylation interference studies of the Flp and γ-δ resolvase site-specific recombination proteins revealed that positions at which adducts blocked recombination corresponded to positions at which adducts blocked recombinase binding *in vitro* (35, 36). In cases where both ethylation interference and x-ray crystallography data are available (for example, 434 repressor and *Eco*RI), both methods have been found to identify common "contacts" with the phosphate backbone (37–40). In this study, the ethylation interference effects in the *target* DNA at the point of strand transfer probably result from interfer-

ence with IN protein binding, because ethylation would be expected to increase the susceptibility of the target phosphate to nucleophilic attack and so might actually promote the strand transfer reaction. The ethylation effects in the viral DNA may be either due to interference with binding to DNA or due to a later reaction step.

The finding that the deletion of two bases from the unjoined strand at the viral DNA terminus specifically impairs strand transfer suggests that subsets of the identified interactions may be important in different stages of the integration process. Contacts to the 2 terminal bases on the unjoined strand may hold the viral DNA end in the proper position for the chemical steps of strand transfer. Alternatively, a single viral DNA end may remain bound to IN protein throughout the terminal cleavage and strand transfer reactions, and this coupling may be disrupted by the 2-base deletion or the interfering primary amine on the unjoined strand. Increased dissociation of the complex after cleavage might diminish strand transfer and increase specific cleavage by freeing IN protein for further cycles of cleavage.

Why is a nearby DNA end necessary for function of IN protein? An abnormal extension of the DNA helix may collide with some required part of the integration apparatus, thereby inhibiting cleavage. Another possibility, not exclusive of the first, is that the DNA end is required to allow IN protein to deform the viral DNA in some manner that is important for function. The energetic cost of deforming DNA near an end is known to be relatively low (41), lending plausibility to this view and highlighting the possibility that viral DNA bound to IN protein may depart from ideal B-form.

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