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Different Roles of Bases within the Integration Signal Sequence of Human Immunodeficiency Virus Type 1 In Vitro

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To investigate the roles of bases near the tips of each strand of the long terminal repeat of the human immunodeficiency virus type 1 in the integration reaction, we examined the efficiencies of both binding and integration activities of staggered-ended substrates and mismatched mutant substrates by the integration assay and the UV cross-linking assay. Our results suggest that some bases of the human immunodeficiency virus type 1 long terminal repeat are required primarily for binding, whereas others are more critical for later reaction steps in vitro.

An essential step in retroviral replication is integration of a linear form of a double-stranded DNA copy of a viral RNA genome into a chromosome of a host cell (1, 11, 12, 19). This integration reaction is catalyzed by the viral integrase (IN) protein with the two ends of the viral DNA as substrate (2, 5, 7, 15, 18). The reaction can be divided into several steps: binding of IN to substrate DNA, 3′ processing, cutting and joining (strand transfer), and gap repair. The last step may be carried out by cellular repair enzymes (for recent reviews of the integration mechanism, see references 13, 25, and 29).

By employing recombinant IN protein and oligonucleotides that mimic the viral DNA ends, in vitro integration systems have been developed (3, 7, 15, 16, 22, 28). By using these systems, DNA sequence requirements and spacing requirements for integration reactions have been revealed (3, 17, 21, 27). The sequence requirements of the terminal 2 bases are not strict (7, 20, 21, 27), but the conserved CA nucleotides must be very close to a DNA end (7, 20, 21, 27). When the CA is located more than 6 bases from an end, the substrate is not cut efficiently (27). The terminal 2 nucleotides of the 3′ end of the plus strand of the U5 long terminal repeat (LTR) are not necessary for the integration reaction in vitro. The efficiency of this precut substrate is higher than that of the blunt-ended substrate (Fig. 1B, lanes 2 and 4) (also see reference 30). On the contrary, the terminal 2 nucleotides of the 5′ end of the minus strand of the U5 LTR are not necessary for the cutting reaction but are required for an efficient joining reaction (at least 1 nucleotide) (4, 21, 27). The next 2 bp, 5′-CA-3′ on the plus strand and 5′-TG-3′ on the minus strand, which are conserved in the sequence of the termini of transposable elements and all retrotransposons, are the most important for the integration reaction. These 2 bp have a critical effect on a sequence-specific binding step (30). The G/C base pair at the fifth position from the U5 LTR end affects both binding and joining activities. The A/T base pair at the sixth position from the U5 LTR end affects both binding and cutting activities. The terminal 6 bp are sufficient for an almost maximum-level integration activity. The sequence from the 11th to the 13th base pair also affects the efficiencies of the cutting and joining reactions to some extent (3, 17, 21, 27), but the methylation of the T residues at the 7th, 9th, and 11th positions on the plus strand of the U5 LTR did not influence the integration activity (4). These results suggest that IN is not in direct contact with the whole terminal 13 bp. Therefore, the terminal 6 bp, which are sufficient for an almost maximum-level integration activity, can be designated as the integration signal sequence (ISS) of the human immunodeficiency virus type 1 (HIV-1) integration reaction.

Recently, we have developed a binding assay by applying a shortwave UV cross-linking method (6) to the in vitro integration system (a UV cross-linking assay) and investigated the binding characteristics of IN with substrate DNA (30). Our results suggest that the sequence- and strand-specific bound state exists at the early phase of the integration reaction before the chemical steps of the 3′ processing reaction and that the IN binding ability of a substrate usually correlates with integration efficiency. Other groups have also reported the results of UV cross-linking assays (10, 26). Engelman and coworkers reported that the core and carboxyl-terminal domains of HIV-1 IN contributed to nonspecific DNA binding (10). Vink and coworkers reported that whole HIV-1 IN was necessary for the formation of a sequence-specific complex between IN and viral DNA (26). Ellison and Brown detected the stable unprocessed DNA integrase complex and the stable processed DNA integrase complex by a modified integration assay (8).

In this paper, we investigated both the integration efficiency and the IN binding ability of staggered-ended substrates and mismatched mutant substrates and analyzed the roles of bases in the HIV-1 ISS.

The plus-strand oligonucleotides of wild-type (WT) and mutant substrates at the U5 end were labeled at the 5′ end with [γ-^{32}P]ATP (6,000 Ci/mmol; Dupont Co.) and T4 polynucleotide kinase (Takara Shuzo Co.). After heating at 85°C for 15 min, a fourfold excess of the unlabeled matched or mismatched complementary strand was added and allowed to anneal by slow cooling to room temperature. HIV-1 IN protein was expressed in SF9 cells infected with a recombinant baculovirus (561-3) and partially purified as previously described (5, 23). Reaction mixtures (30 μl) for both the integration assay and the UV cross-linking assay were the same and contained 34 mM MOPS (morpholinepropanesulfonic acid) (pH 7.2), 80 mM potassium glutamate, 15 mM $MnCl_2$, 10 mM 2-mercaptoethanol, 9% glycerol, 10% dimethyl sulfoxide, 0.1 g of bovine serum albumin per ml, 10 ng of labeled synthetic oligonucleotide substrates, and about 90 ng of the HIV-1 IN protein. Reaction mixtures were incubated at 30°C for 5 min, and half of each mixture was spotted onto Parafilm (American National Can) and irradiated from 2.5 cm above with a UV transillumi-

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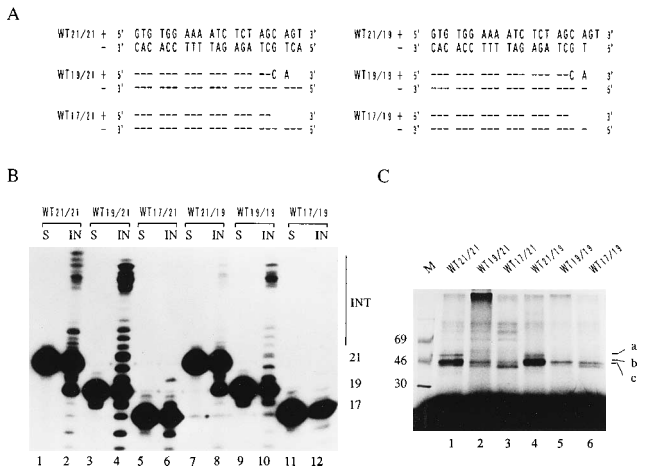

FIG. 1. Integration and photoadduct formation efficiencies of staggered-ended substrates. (A) Sequences of substrates. All substrates were labeled at the 5′ end of the plus strand (indicated by a plus sign). (B) Analysis of integration reaction products on a sequence gel. Each lane contains the substrate marked above the lane. Lanes S, no HIV-1 IN lysate; lanes IN, 90 ng of HIV-1 IN lysate. The positions of integration products are indicated (INT). (C) Analysis of the photoadducts by autoradiography of the SDS-polyacrylamide gel of the UV cross-linked integration reaction mixtures. Each lane contains the same reaction mixture as that in panel B but UV irradiated. The positions of each of the three photoadduct bands are indicated (a, b, and c). Lane M, ^{14}C-methylated protein molecular weight markers (NEN) (molecular weight in thousands is indicated at left of panel C).

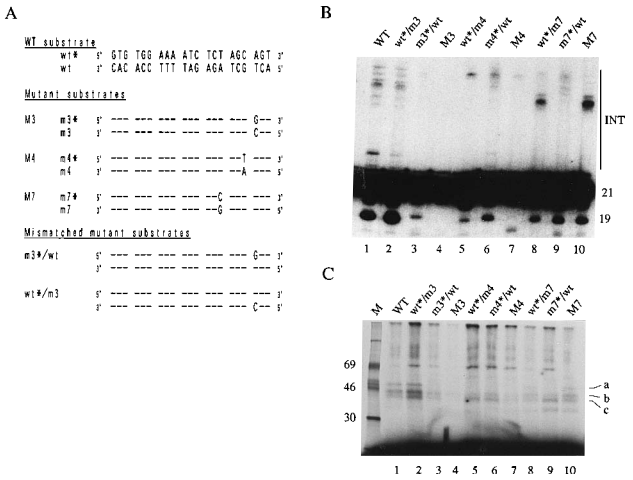

FIG. 2. Integration and photoadduct formation efficiencies of mismatched mutant substrates. (A) Sequences of WT, mutant, and mismatched mutant substrates. Only two mismatched mutant substrates are shown. All substrates were labeled at the 5′ end of the plus (indicated by an asterisk) strand. (B) Analysis of integration reaction products on a sequence gel. Each lane contains the substrate marked above the lane. The positions of integration products are indicated (INT). (C) Analysis of the photoadducts by autoradiography of the SDS-polyacrylamide gel of the UV cross-linked integration reaction mixtures. Each lane contains the same reaction mixture as that in panel B but UV irradiated. The indications of photoadduct bands and protein molecular weight markers (lane M) (molecular weight in thousands is indicated at left) are the same as in Fig. 1.

nator (wavelength, 254 nm) (model UVG-54; Mineralight Lamp) at room temperature for 10 min. After heating at 90°C for 3 min, the mixtures were loaded onto a sodium dodecyl sulfate (SDS)–9% polyacrylamide gel and analyzed by autoradiography. The remainder of the reaction mixtures were incubated at 30°C for 30 min and stopped by the addition of 15 μl of loading buffer (95% formamide, 20 mM EDTA [pH 8.0], 0.05% bromophenol blue, 0.05% xylene cyanol). The samples were heated for 2 min at 85°C, and 5-μl aliquots were electrophoresed on a denaturing 20% polyacrylamide gel. Autoradiography was performed by exposing the wet gel to X-ray film (X-OMAT AR, Eastman Kodak Co., or HR film, Fuji Photo Film Co.) at −80°C for overnight or longer.

In order to investigate the roles of bases in the HIV-1 ISS, we made a series of staggered-ended substrates (Fig. 1A). When the plus strand was a 19-mer and the minus strand was a 21-mer ($WT_{19/21}$ or WT_{precut}), the integration efficiency was higher than that of $WT_{21/21}$ (or $WT_{blunt\text{-}end}$) as previously described (Fig. 1B, lanes 2 and 4) (also see reference 30). However, the substrate with a shorter plus strand ($WT_{17/21}$) did not exhibit any integration activity at all (Fig. 1B, lane 6). On the other hand, when the minus strand was a 19-mer ($WT_{21/19}$), the joining activity was greatly diminished but the cutting activity was similar to that of $WT_{21/21}$ (Fig. 1B, lanes 2 and 8). This result is very interesting because these terminal 2 nucleotides of the minus strand are not present in the final integration product. They are eventually eliminated during the repair synthesis (4, 21, 27). Moreover, the integration efficiency of $WT_{19/19}$ substrate was several times lower than that of $WT_{19/21}$ substrate (Fig. 1B, lanes 4 and 10). $WT_{17/19}$ substrate did not integrate at all (Fig. 1B, lane 12).

Next, we investigated the binding efficiencies of the same substrates by a UV cross-linking assay (Fig. 1C). In this assay, three different bands are observed with the blunt-end substrate (Fig. 1C, lane 1) as previously shown (30). The differences in the sizes of these photoadducts were not due to the size of IN protein itself, because we could not detect any proteolytically processed IN protein corresponding to the size of band b or c on the SDS-polyacrylamide gel by Coomassie brilliant blue staining (data not shown). $WT_{19/21}$ substrate did not form band a, although it had a very high integration activity, because band a was derived from a precleavage complex (Fig. 1C, lane 2) (also see reference 30). It formed both band b and band c (Fig. 1C, lane 2). $WT_{17/21}$ substrate, which did not have the integration activity, formed band c (Fig. 1C, lane 3). These data also suggest that band c is derived from a complex in which IN bound to DNA nonspecifically. The important observation was that $WT_{21/19}$ substrate formed all three photoadducts (Fig. 1C, lane 4), although it showed only very low joining activity (Fig. 1B, lane 8). The intensities of these bands were almost the same as those of the $WT_{21/21}$ substrate (Fig. 1C, lanes 1 and 4). This result indicates that the terminal 2 nucleotides from the 5′ end of the minus strand do not play a critical role in the formation of complexes between IN and DNA or the 3′ processing of the plus strand but play an important role in the joining step. Formation of band b with the $WT_{21/19}$ substrate may be the result of accumulation of initial binding complexes and a certain type of postcleavage complex because this substrate cannot proceed to strand transfer. $WT_{19/19}$ substrate formed bands b and c faintly (Fig. 1C, lane 5), and $WT_{17/19}$ substrate formed bands b and c (Fig. 1C, lane 6). Formation of band a with $WT_{21/19}$ substrate and not with $WT_{19/19}$ or $WT_{17/19}$ correlates with our view that band a is derived from an early phase of the integration reaction before the 3′ processing reaction (precleavage complex).

Furthermore, we investigated the roles of bases in the HIV-1 ISS by comparing WT substrate, mutant substrates, and mismatched mutant substrates. The sequences of these mutant substrates are shown in Fig. 2A. While mutant substrate m3*/m3 (M3) is neither cut nor joined (Fig. 2B, lane 4), mismatched mutant substrate wt*/m3, which has the WT sequence on the plus strand, recovered the activities of both cutting and

joining (Fig. 2B, lane 2). The cutting products of wt*/m3 substrate were more abundant than those of WT substrate, and the joining products of it were less abundant than those of WT substrate (Fig. 2B, lanes 2 and 1). This suggests the similar efficiency of cutting and low efficiency of strand transfer of this substrate compared with WT substrate. On the other hand, the m3*/wt substrate also recovered both activities, but the cutting activity was low and the joining activity was very low (Fig. 2B, lane 3). In the case of the m4 mutants, while mutant substrate m4*/m4 (M4) was almost neither cut nor joined (Fig. 2B, lane 7), both substrates which have the WT sequence on only one of the strands recovered the activities of both cutting and joining (Fig. 2B, lanes 5 and 6), but less than WT substrate. The joining activities of the two m4 mismatched substrates were almost the same, but the cutting activity of the m4*/wt substrate was more efficient than that of the wt*/m4 substrate (Fig. 2B, lanes 5 and 6). Both activities of the wt*/m7 mismatched substrate were slightly less than those of mutant substrate m7*/m7 (M7) (Fig. 2B, lanes 8 and 10). The cutting activity of m7*/wt was the same as that of M7 mutant substrate (Fig. 2B, lanes 9 and 10). The joining activity of m7*/wt was slightly less than that of M7 mutant substrate, and the pattern of integrated products was changed. This probably results from an effect of target DNA conformation on recognition by IN as was pointed out previously (14). We examined other mismatched mutant substrates, m5, m6, and m11 to -13 (data not shown); both the activities were not influenced critically. Therefore, these results with m3 and m4 mismatched mutants are not artifacts.

In order to examine the binding activities of these substrates, we performed UV cross-linking assays under the same conditions as the integration assays (Fig. 2C). WT substrate formed three photoadduct bands (Fig. 2C, lane 1). M3 substrate had no integration activity and formed only band b very faintly (Fig. 2C, lane 4). Interestingly, wt*/m3 substrate formed all three bands more abundantly than WT substrate (compare Fig. 2C, lanes 1 and 2). IN seemed to join the wt*/m3 substrate with slower kinetics than WT substrate, and the cut products accumulated (Fig. 2B, lanes 1 and 2). The m3*/wt substrate formed more of both bands b and c than M3 substrate but less than WT substrate (Fig. 2C, lane 3). M4 substrate formed both bands b and c very faintly (Fig. 2C, lane 7). The wt*/m4 substrate formed band c more than WT substrate and formed band b faintly (Fig. 2C, lane 5). The m4*/wt substrate showed almost the same pattern as wt*/m4 (Fig. 2C, lane 6). Both the m4 substrates did not form band a. These results suggest that both bases at the fourth position are important for the precleavage complex formation. Both the m4 substrates formed band c more strongly than M4 substrate. We do not know why m4 mismatched substrates formed band c more abundantly than M4 substrate. One possibility is that the reversion to the WT base of either strand of M4 substrate recovered postcleavage complex formation, which facilitated the binding of target DNA to form band c. This possibility is worth examining later. Both wt*/m3 and wt*/m4 substrates had the WT plus strand, but the results of UV cross-linking assay of these substrates were very different. These results suggest that the G residue at the fourth position from the 5′ end of the minus strand is very important for the sequence-specific binding of IN and substrate DNA and that the importance of the G residue for the overall integration reaction is higher than that of the T residue in the third position of the minus strand. On the other hand, when the minus strand is WT, mutation at the third position of the plus strand affects the overall integration efficiency more than that at the fourth position. M7 substrate formed all three bands as well as WT substrate (Fig. 2C, lane 10). The wt*/m7 substrate also formed three bands but less than WT substrate

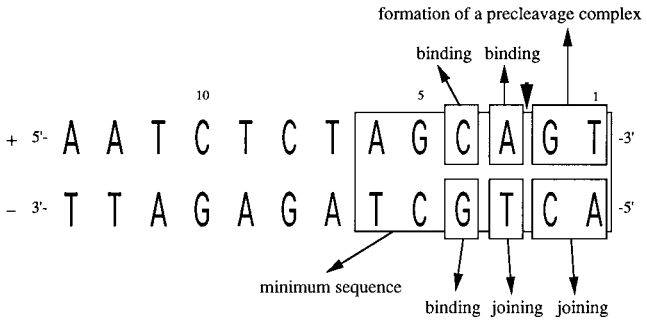

FIG. 3. Summary of the roles of bases of HIV-1 ISS of U5 LTR in the integration reaction in vitro. The terms in the figure are as follows. Minimum sequence: a substrate which has the terminal 6 bp shows almost the maximum level of integration activity; binding: a change of the base affects a sequence-specific binding step; joining: a change of the base or a removal of the base affects joining activity; formation of a precleavage complex: though these nucleotides are not necessary for integration reaction, their removal is necessary for the reaction, and then IN forms a precleavage complex with these nucleotides. A small arrowhead indicates the point of 3′ processing.

(Fig. 2C, lanes 8 and 1). The m7*/wt substrate formed bands b and c and formed band a faintly (Fig. 2C, lane 9). Band c of the m7*/wt was more abundant than that of WT. The formation of band c may also be affected by the efficiency of strand transfer. We also examined other mismatched mutant substrates, m5, m6, and m11 to -13 (data not shown). These mismatched mutant substrates affect the efficiencies of formation of complexes to some extent, but no big difference was observed among matched and mismatched mutants. Compared with the above results, the effects of m3 and m4 mismatched substitution are evident. Therefore, the different roles and relative importance of bases in the ISS are shown.

We have already suggested that there exists a correlation between the integration activity and the DNA binding activity and that M3 and M4 substrates are neither cut nor joined mainly because these are not bound by IN (30). We have shown in this paper that 8 nucleotides in the terminal 4 bp of the HIV-1 ISS have different roles in the integration reaction (Fig. 3). The terminal 2 nucleotides of the plus strand play an important role in the 3′ processing step by forming a precleavage complex, although these nucleotides are not necessary for the integration reaction (Fig. 1B, lanes 2 and 4, and Fig. 1C, lanes 1 and 2). Three residues, the A residue at the third position from the 3′ end of the plus strand, the C residue at the fourth position from the 3′ end of the plus strand, and the G residue at the fourth position from the 5′ end of the minus strand, are very important for the substrate binding. Among these, the A residue at the third position from the 3′ end of the plus strand is the most important, and the second most important residue is the G residue at the fourth position from the 5′ end of the minus strand (Fig. 2C). The T residue at the third position from the 5′ end of the minus strand is necessary not for binding and 3′ processing steps but for the maximum level of joining activity. The existence of the terminal 2 nucleotides of the minus strand is important in the joining step even though by a sequence-independent manner. Ellison and Brown showed that a complex between IN and ΔAC viral DNA substrate (equivalent to $WT_{21/19}$ substrate) was unstable compared with the standard substrate (equivalent to $WT_{21/21}$ substrate) (8). This may be one of the reasons for the low joining activity of $WT_{21/19}$, although we do not know whether their stable processed complex is the same as our postcleavage complex. Considering that three nucleotides which are necessary for the maximum level of joining activity are lined up along the

end of the minus strand of the U5 ISS (Fig. 3), we propose a new model of the HIV-1 IN integration mechanism expanded from our previous model (30). First, IN binds substrate DNA in a sequence-independent manner (an initial binding complex). Second, IN searches for the ISS at the DNA end and binds tightly in a sequence-specific manner to form the precleavage complex. Third, IN processes 2 nucleotides at the 3′ end of plus strand of ISS. Fourth, a part of IN grasps the terminal 3 (at least 2) nucleotides of the minus strand and another part of IN grasps the terminal 4 nucleotides (5′-AGCA-3′) of the plus strand to form the postcleavage complex. In this complex, the recessed 3′ ends of both termini of viral DNA are correctly positioned for the concerted integration reaction. This IN-substrate DNA complex may be stable when there is no available target DNA. In this complex, we suppose that the terminal parts of each strand of both ends of viral DNA are separated, because we have preliminary data that each strand bound another part of IN (29a). Fifth, target DNA binds to this postcleavage complex. Sixth, the DNA strand transfer reaction proceeds. Seventh, IN dissociates from the product before the gap repair (30).

In this paper, we focused on band a especially because this is derived from a precleavage complex and apparently a sequence-specific one. Band b is not a single photoadduct (30) and is difficult to define clearly. But we think that band b may consist of various photoadducts which derived from an initial complex, a precleavage complex (only when the minus strand of substrate DNA is UV cross-linked), and a postcleavage complex. Band c is derived from a complex in which IN binds target DNA in a sequence-independent manner.

Recently, Van Den Ent and coworkers investigated the integration activity of mismatched mutant substrates (heteroduplex substrates) (24). Their results were similar to our results, but they did not distinguish between the DNA binding activity and the integration activity.

We believe that our findings will be useful for further investigation of the interaction between IN and DNA and for the rational design of an IN inhibitor.

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