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Role of the His-Cys Finger of Moloney Murine Leukemia Virus Integrase Protein in Integration and Disintegration

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Retroviral integrases mediate site-specific endonuclease and transesterification reactions in the absence of exogenous energy. The basis for the sequence specificity in these integrase-viral DNA recognition processes is unknown. Structural analogs of the disintegration substrate were made to analyze the disintegration reaction mechanism for the Moloney murine leukemia virus (M-MuLV) integrase (IN). Modifications in the target DNA portion of the disintegration substrate decreased enzymatic activity, while substitution of the highly conserved CA in the viral long terminal repeat portion had no effect on activity. The role of the His-Cys finger region in catalysis was addressed by *N*-ethylmaleimide (NEM) modification of the cysteine residues of M-MuLV IN as well as by mutations. Both integration activities, 3′ processing, and strand transfer, were completely inhibited by NEM modification of M-MuLV IN, while disintegration activity was only partially sensitive. However, structural analogs of the disintegration substrates that were modified in the target DNA and had the conserved CA removed were not active with NEM-treated M-MuLV IN. In addition, mutants made in the His-Cys region of M-MuLV IN were examined and found to also be completely blocked in integration but not disintegration activity. These data suggest that the domains of M-MuLV IN that are required for the forward integration reaction substrate differ from those required for the reverse disintegration reaction substrate.

Establishment of the proviral state by retroviruses is accomplished by the integrase (IN) protein and is an essential step in the retroviral life cycle (10, 25, 29, 46). After reverse transcription of the viral RNA, IN specifically recognizes the long terminal repeat (LTR) at each viral DNA end and removes the two terminal nucleotides 3′ to the highly conserved dinucleotide sequence CA (3′ processing) (3, 26, 36, 42). The second catalytic step, strand transfer, is an isoenergetic transesterification reaction (15) whereby IN mediates a nucleophilic attack with each 3′-hydroxyl moiety at the processed viral ends to a 5′-phosphate in the host DNA (2, 10, 16, 17, 25, 48). Strand transfer by Moloney murine leukemia virus (M-MuLV) IN joins each viral end to sites in opposing DNA strands that are separated by four bases. The single-stranded gaps adjacent to each 5′ unligated viral strand are subsequently filled in, thereby creating a direct repeat sequence in the host DNA. Both reactions of retroviral integration (3′ processing and strand transfer) are proposed to occur by a one-step mechanism that does not involve a covalent intermediate between integrase and DNA (15, 33).

The reverse of the integration process or disintegration has been demonstrated in vitro with purified human immunodeficiency virus type 1 (HIV-1) integrase (6, 41) and M-MuLV integrase (23). The reaction is characterized by the site-specific excision of one viral end that is joined to a host target sequence. The endonucleolytic cleavage occurs specifically after the conserved 5′-CA-3′ in the LTR, and the host target sequence is closed by integrase. Neither the integration nor the disintegration process requires an exogenous energy source for in vitro reactions; however, all reactions do require Mn^{2+} or Mg^{2+} (2, 6, 10, 23, 25).

The active conformation that retroviral integrases assume in these biochemical processes has been addressed through biophysical and kinetic analysis of avian myeloblastosis virus IN (19), Rous sarcoma virus (RSV) IN (22), and (HIV-1) IN (47). Zonal centrifugation studies show that RSV IN is able to form dimeric and tetrameric conformations, while HIV-1 and avian myeloblastosis virus IN proteins sediment as dimers. Kinetic analysis of the RSV IN indicates that multimerization is important for optimal integration activity. The features of integrase required for the observed oligomerization are unknown.

The protein domain(s) of IN that mediates the recognition of viral and host DNA and the subsequent binding and catalytic steps are unknown. Retroviral integrases contain two highly conserved regions, a putative zinc finger motif in the amino terminus (21) and a central region that features three amino acids essential for integration and disintegration activity consisting of two aspartic acids and a glutamic acid (13, 14, 18, 27, 28, 31). Structural analysis of the putative zinc finger region in HIV-1 IN shows that His-12, Cys-40, and Cys-43 coordinate Zn^{2+} (4). The function of the zinc finger domain has been addressed by mutational analysis of these conserved His and Cys residues in several retroviral integrases. Mutations in the zinc finger domain (His-X_3-His-X_{30}-Cys-X_2-Cys) of M-MuLV IN reconstructed in the virus in vivo are not viable (11, 37). However, some of these same mutations analyzed in recombinantly expressed M-MuLV IN do retain a nonspecific DNA binding activity in vitro (37). Deletions have been made in the finger region of avian sarcoma and leukosis virus (27), RSV (34), and HIV-1 (40, 51) integrases. These IN proteins also retain nonspecific DNA binding ability that is attributed to the carboxyl-terminal half. Hence, the finger region is proposed to be involved in the recognition of the retroviral LTR termini (27), correct binding and positioning of the LTR end (18), or protein-protein interactions (14). Mutations of the His and Cys residues in the finger region of HIV-1 (14, 31, 47) and HIV-2 (18) IN, in general, greatly decrease the 3′-processing

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and strand transfer activities but have minimal effect on disintegration activity.

M-MuLV IN has three cysteine residues, two located in the His-Cys finger region and the third cysteine located in the central core region. We have chemically modified the cysteine residues in M-MuLV IN to probe the role of the His-Cys finger in integration and disintegration activities. Mutations made in the His-Cys region that are inactive in vivo (37) were also examined for the individual reactions of integration and disintegration activities in vitro. Furthermore, structural derivatives of the disintegration substrate were made to address what is being recognized by M-MuLV IN. Based on our results, a preliminary model on the interaction of M-MuLV IN with the viral LTR substrates in integration and disintegration reactions is proposed. The working model proposes a single catalytic domain, yet the recognition mediated by integrase-DNA interactions within this site is distinct between integration and disintegration. These differences in substrate recognition support a minimum of two domains in M-MuLV IN that interact with DNA, the zinc finger region and a nonspecific target DNA region. The 3′-processing and strand transfer activities require contact with the putative zinc finger or a conformation provided by this structure, which is necessary for integration activity.

MATERIALS AND METHODS

Oligonucleotide substrate preparation. DNA oligonucleotides were prepared on an Applied Biosystems model 380B DNA synthesizer by the UMDNJ Biochemistry DNA synthesis facility. Oligonucleotides were purified and 5′ end labeled with [γ-^{32}P]ATP (ICN, Irvine, Calif.), using T4 polynucleotide kinase (GIBCO-BRL) as previously described (23). Oligonucleotide substrates that mimic the U5 LTR were prepared by end labeling the 5′ end of oligonucleotide 2783 (5′-GTCAGCGGGGGTCTTTCATT) for the 3′-processing reaction or the 5′ end of oligonucleotide 2784 (5′-GTCAGCGGGGGTCTTTCA) for the strand transfer reaction. The labeled strands were hybridized to a fivefold molar excess of unlabeled oligonucleotide 2785 (5′-AAT GAAAGACCCCCGCTGAC) in 100 mM NaCl. Unincorporated label was removed by G-25 Quick Spin columns (Boehringer Mannheim).

Two sets of disintegration substrates were made and will be referred to by the B-strand oligonucleotide number (see Fig. 2A). The Y3403 series is composed of A-strand 3415 (5′-AATGAAAGACCCCACCAGAAG), B-strand 3403 (5′-CTTCTGGTGGGGTCTTTCACCATAGTCAACACG), C-strand 3407 (5′-ATCACCGCCAGAGGTA), and D-strand 3398 (5′-CGTGTTGACTATGGTACCTCTGGCGGTGAT). The 3901 series is composed of B-strand 3901 (5′-CTTCTG GTGGGGTCTTTGTCCATAGTCAACACG), A-strand 3902 (5′-AAACAAAGACCCCACCAGAAG), C-strand 3407, and D-strand 3398. Disintegration substrates were hybridized and gel purified as previously described (23).

Construction of M-MuLV integrase mutants. The four-amino-acid linker insertion Arg-Asn-Ser-Gly, positioned between Ser-63 and Phe-64, and the single-amino-acid mutation Cys-95→Tyr (C95Y) were constructed as previously described (37). These mutations were reconstructed into pETINH1 (23) by simple replacement of an *Acc*I fragment (nucleotides [nt] 4719 to 5968 of the viral RNA sequence (43). The nucleotide sequence of each mutation was verified by dideoxynucleotide chain terminator sequencing (39) (United States Biochemical).

Purification of M-MuLV integrase. Recombinant M-MuLV integrase containing a hexahistidine tag at the carboxyl terminus was expressed in *Escherichia coli* BL21DE3 (Novagen) and purified by a one-step method on nickel nitriloacetate affinity resin as previously described (23). Mutant integrase proteins were similarly expressed and purified. Protein concentration was measured by the method of Bradford (1) (Bio-Rad).

Disintegration assays. Endonucleolytic cleavage of the B strand in the Y substrate and joining of the B and C strands were assayed at 30°C for 1 h or overnight in a 15-μl volume containing 0.2 pmol of substrate, 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 5 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate), 10 mM dithiothreitol (DTT), 25 mM $MnCl_2$, 0.05% Nonidet P-40, and 1 to 5 pmol of IN protein. Reactions were stopped by the addition of 10 μl of stop buffer (95% formamide, 20 mM EDTA, 0.5% bromophenol blue, 0.5% xylene cyanol). The B strand was ^{32}P labeled at the 5′ end to monitor the endonucleolytic cleavage, as was the C strand for joining. Reaction products were separated on 20% denaturing (8.3 M urea) polyacrylamide gels that were dried and exposed to X-ray film at −70°C or PhosphorImager screens for 30 min to overnight. Images were scanned with a PhosphorImager (Molecular Dynamics, Sunnydale, Calif.), and ImageQuant 3.15 software was used to quantitate data from individual reactions.

3′-Processing and strand transfer assays. Standard integration reaction mixtures for 3′-processing and strand transfer activities contained 1 pmol of 2783/2785 or 2784/2785 substrate, 20 mM 4-morpholineethanesulfonic acid (MES; pH 6.2), 10 mM $MnCl_2$, 10 mM DTT, and 1 to 5 pmol of IN. Reaction mixtures (15 μl) were incubated for 1 h at 30°C, and reactions were stopped with 10 μl of stop buffer.

NEM assays. Three separate preincubations, M-MuLV IN on ice, M-MuLV IN with 10 or 20 mM *N*-ethylmaleimide (NEM), and M-MuLV IN with 50 mM DTT, were made on ice for 30 min prior to integration or disintegration assays as described above. After incubation for 30 min on ice as indicated, DTT was added to a final concentration of 50 mM to M-MuLV IN preparations treated with 10 mM NEM. Likewise, NEM was added to a final concentration of 10 mM to IN preparations treated with 50 mM DTT. The preincubated M-MuLV IN (1 to 5 pmol) was then added to reaction mixtures as described above for integration and disintegration assays and incubated for 1 h or overnight at 30°C.

RESULTS

Recognition of disintegration substrate by M-MuLV integrase. An illustration of the integration and disintegration reactions catalyzed by M-MuLV IN is presented in Fig. 1. The enzymatic activities of IN are assayed in vitro, using oligonucleotide substrates representative of one viral LTR end that are labeled at the 5′ end with ^{32}P. In Fig. 1, reaction steps 1 and 2 comprise the forward integration reactions, which result in the formation of a Y substrate (6). The two products of disintegration are shown as steps 3 and 4. The assay for the site-specific endonucleolytic cleavage (endonuclease activity) requires that the B strand be ^{32}P labeled, so that correct cleavage of the LTR-target DNA junction on the B strand generates a 19 bp ^{32}P-labeled product (Fig. 1, step 3). The second activity of disintegration, joining, is the sealing of the nick in the target DNA strand of the Y substrate that results in a 30-nt product (Fig. 1, step 4).

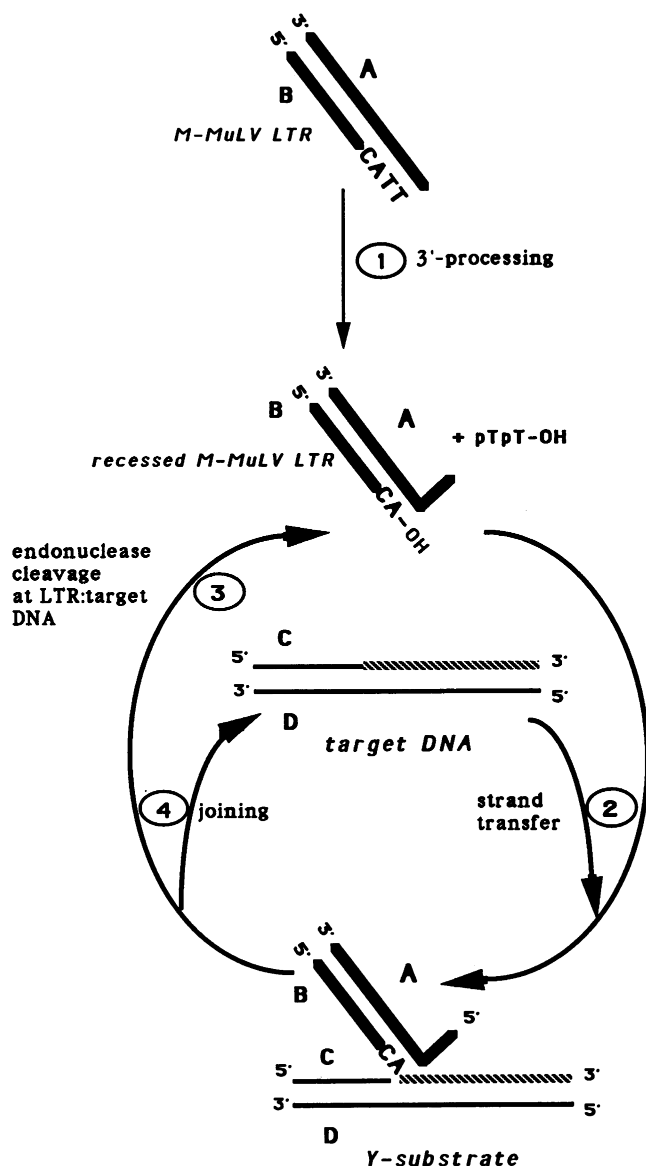


FIG. 1. Illustration of the biochemical reactions mediated by M-MuLV IN in vitro. The substrate and products of integration (reactions 1 and 2) and disintegration (steps 3 and 4) are detected by radiolabeling one strand of the substrate being examined. The radiolabeled substrate is incubated at 30°C with M-MuLV IN; the resulting reaction products are separated on a 20% denaturing polyacrylamide gels and subjected to autoradiography as described in Materials and Methods. The first step in integration is the 3' processing that occurs specifically 3' to the conserved CA of the viral LTR (reaction 1). This dinucleotide cleavage is detected by ^{32}P labeling of 5' end of the B strand (20 nt). Cleavage of the labeled strand results in the generation of an 18-nt product upon incubation with M-MuLV IN. The next step, strand transfer, is a transesterification reaction mediated by IN which results in ligation of the 3' end of the cleaved B strand to the 5' end of the target (reaction 2). Radiolabeled products of this reaction are larger and smaller than the starting substrate. Disintegration consists of a cleavage between the conserved LTR and target DNA junction of what is now referred to as the B strand (step 3). The final activity shown (joining; step 4), is the closing of the nick between the target sequences at the B- and C-strand junction. Detection of this product is made by ^{32}P labeling of the C strand (16 nt), which when joined to the B strand by M-MuLV IN generates a 30-bp product.

M-MuLV IN joining activity is followed by 5' end labeling the C strand (16 nt).

To determine the features of the disintegration substrate recognized by M-MuLV IN, structural derivatives of the wild-type M-MuLV IN substrate Y3403 were made (Fig. 2A, top line). Briefly, Y3403dnt has only the 3'-terminal nucleotide removed on the C strand, while Y3403dC is missing the C strand entirely. In addition, a second series of substrates was made from the disintegration substrate Y3901, which has the conserved dinucleotide CA replaced with the dinucleotide GT (Fig. 2A, bottom line). Each substrate (Fig. 2A) was assayed for the B-strand endonuclease activity with purified M-MuLV IN. As shown previously (23), M-MuLV IN efficiently releases the 19-bp viral sequence from the wild-type disintegration substrate, Y3403 (Fig. 2B, lane 2). Substitution of the CA with a GT in substrate Y3901 displayed no difference compared with Y3403 in the amount of the 19-mer product accumulated (Fig. 2B, lane 8). In contrast, in similar experiments with HIV-1 IN, substitution of the CA results in a marked decrease in activity (6). These results suggest that M-MuLV IN relies on features outside of the conserved CA to correctly cleave the B strand. M-MuLV IN was also able to correctly cleave a Y-oligomer substrate containing the HIV-1 LTR (12).

The first modification of the Y substrate was removal of the 3'-terminal nucleotide on the C strand (substrate Y3403dnt; Fig. 2A). Removal of the terminal nucleotide on the C strand (Fig. 2B, lane 4) reduced cleavage activity 77% in comparison with the wild type, Y3403. A further decrease in activity was seen with the same modification of Y3901, Y3901dnt, which had 10% of the wild-type activity of its parent substrate, Y3901 (Fig. 2B, lane 10). Therefore, the effect of the GT substitution and the removal of the 3'-terminal nucleotide were synergistic in the reduction of M-MuLV IN disintegration activity.

The second alteration of the Y substrate was removal of the C strand, which should alter the Y structure considerably. Elimination of the C strand in the wild-type substrate, Y3403dC (Fig. 2B, lane 6), reduced activity 77%, a percent reduction similar to that found for Y3403dnt. Both of these substrates lack the terminal 3'-OH of the C strand at the Y junction. M-MuLV IN recognized these structurally different substrates in similar fashions, which could indicate that the terminal 3'-OH of the C strand assists in positioning or stabilizing IN on the target binding site. As noted with Y3901dnt, a dramatic reduction in activity was observed with the GT-substituted substrate, Y3901dC, the activity of which was much less than that of Y3403dC. Y3901dC had only 3% of the activity of the parent substrate, Y3901 (Fig. 2B, lane 12). The results with the C-strand modifications suggest that the target DNA site of the Y oligomer is an important feature for disintegration substrate recognition by M-MuLV IN. Furthermore, if the target DNA site is altered, M-MuLV IN relies on the LTR sequences, such as the CA, to direct the required catalysis.

A longer incubation of these disintegration reactions indicated a further distinction between the Y3403 and Y3901 substrates in terms of activity. Prolonged reactions with the wild-type Y3403 substrate (Fig. 2B, lane 13), Y3403dnt (Fig. 2B, lane 14), and Y3403dC (Fig. 2B, lane 15) with M-MuLV IN continued to accumulate twice the levels of the 19-nt product compared with the 1-h reactions. More remarkably, the released product was efficiently reintegrated into the substrate. The levels of integration attained in reactions with Y3403 and its derivatives were similar. In contrast, reintegration of the 19-bp cleaved product was not observed with

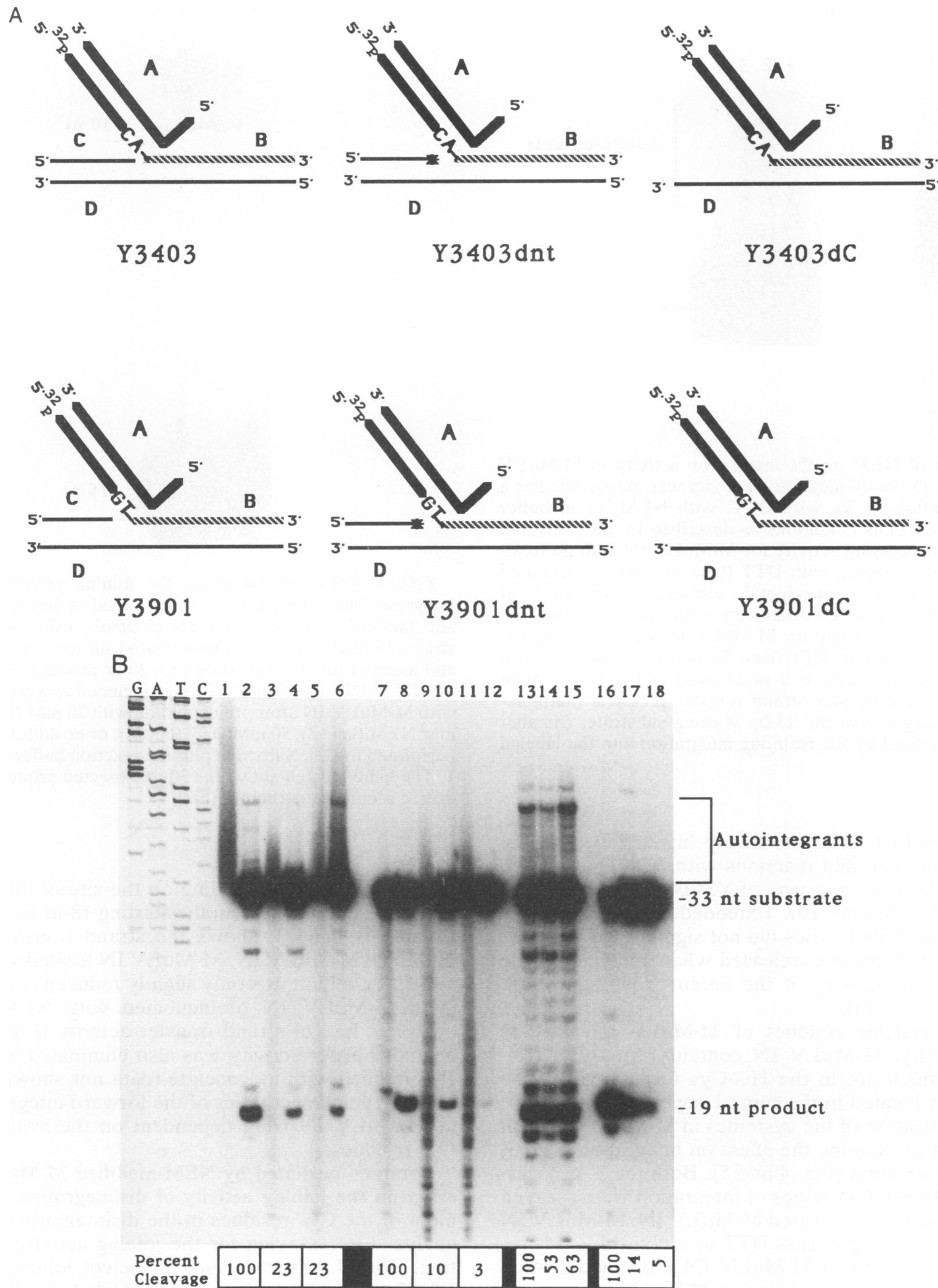


FIG. 2. Endonuclease activity of M-MuLV IN. (A) Illustration of two Y substrates (Y3403 and Y3901) and structural derivations, assayed for endonuclease activity of disintegration with M-MuLV IN. Asterisks in substrates Y3403dnt and Y3901dnt denote the absence of one nucleotide. (B) Endonuclease activity was assayed with 0.2 pmol of substrate in the presence of 5 pmol of M-MuLV IN for 1 h (lanes 1 to 12) or overnight (lanes 13 to 18) as described in Materials and Methods. Reactions: Y3403 (lane 1), Y3403 plus IN (lane 2), Y3403dnt (lane 3), Y3403dnt plus IN (lane 4), Y3403dC (lane 5), Y3403dC plus IN (lane 6), Y3901 (lane 7), Y3901 plus IN (lane 8), Y3901dnt (lane 9), Y3901dnt plus IN (lane 10), Y3901dC (lane 11), Y3901dC plus IN (lane 12), Y3403 plus IN (lane 13), Y3403dnt plus IN (lane 14), Y3403dC plus IN (lane 15), Y3901 plus IN (lane 16), Y3901dnt plus IN (lane 17), and Y3901dC plus IN (lane 18). Specific cleavage of the ^{32}P-labeled B strand (33 nt) generates a 19-bp product. The 19-bp product can reintegrate into the Y substrates, creating autointegrants as indicated. Products were separated on a 20% denaturing polyacrylamide gel. Substrate breakdown, observed in 1-h reactions, results in a ladder of radioactive background (lanes 7, 9, and 11). Interestingly, this background of substrate breakdown is never observed in the presence of M-MuLV IN. A DNA sequence ladder (lanes G, A, T, and C) was included as a sizing ladder. The percentage of wild-type endonuclease activity for each substrate is indicated at the bottom.

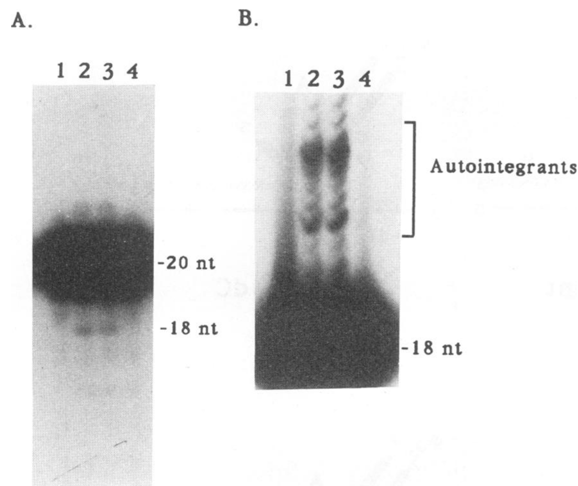


FIG. 3. Effect of NEM on the integration activity of M-MuLV IN. M-MuLV IN (5 pmol) integration activity was measured after a 30-min preincubation on ice with DTT, with NEM, or in buffer under standard reactions conditions as described in Materials and Methods. (A) 3′-processing activity for M-MuLV IN with no treatment (lane 2), preincubated with DTT (lane 3), and preincubated with NEM (lane 4). The dinucleotide cleavage yields an 18-nt product from a 20-nt substrate (indicated at the right; see also Fig. 1). (B) Strand transfer activity for M-MuLV IN with no treatment (lane 2), preincubation with DTT (lane 3), and preincubation with NEM (lane 4). The substrate is a precleaved DNA (Fig. 1) with which M-MuLV IN generates strand transfer products (autointegrants) that are larger than the 18-bp starting substrate. (Smaller products are generated by the resulting integration into the labeled strand.)

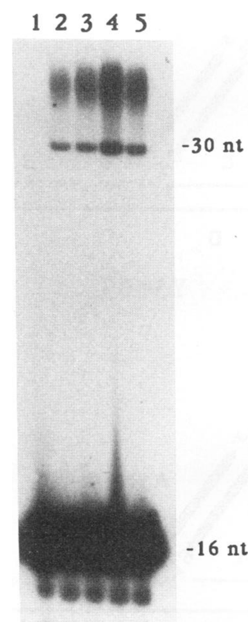


FIG. 4. Effect of NEM on the joining activity of M-MuLV integrase. The joining activity was assayed as described in Materials and Methods with M-MuLV IN (2 pmol), using Y3403 as a substrate. M-MuLV IN was preincubated on ice alone or with NEM and assayed for the formation of a 30-nt product. Preincubation of M-MuLV IN with 50 mM DTT was included as a control. Reactions with M-MuLV IN after preincubation with 20 mM NEM (lane 2), 10 mM NEM (lane 3), 50 mM DTT (lane 4), or no addition (lane 5) were performed for 1 h. Substrate plus the reaction buffer is shown in lane 1. The band located above the 30-nt expected product is reportedly due to secondary structure (6).

Y3901. This resulted in quantitatively higher levels of the 19-bp product in overnight reactions with Y3901, as can be observed by a direct comparison of Y3403 (Fig. 2B, lane 13) and Y3901 (Fig. 2B, lane 16). Extended incubation of the derivatives of the Y3901 series did not significantly increase the amount of 19-nt product released when analyzed as the percentages of the activity of the parent Y3901 substrate (Fig. 2B, lanes 16 to 18).

Role of the cysteine residues of M-MuLV integrase in integration activity. M-MuLV IN contains three Cys residues, two of which are in the His-Cys finger region. The third cysteine is located in the central region of the protein. Chemical modification of the cysteines in M-MuLV IN with NEM was used to examine the effect on integration (Fig. 3) as well as disintegration (Fig. 4 and 5). Both the 3′-processing and strand transfer activities of integration were assayed with NEM-treated and untreated M-MuLV IN. M-MuLV IN was also incubated with excess DTT as a control.

3′-processing activity of M-MuLV IN after modification with NEM was assayed by using a 20-bp blunt substrate (Fig. 1, step 1). M-MuLV IN specifically removed the two terminal nucleotides from the 3′ end of the ^{32}P-labeled strand. The 18-nt product of the 3′-processing reaction was readily detected for untreated (Fig. 3A, lane 2) and DTT-pretreated (Fig. 3A, lane 3) M-MuLV IN. However, preincubation of M-MuLV IN with NEM resulted in complete blockage of the 3′-processing activity (Fig. 3A, lane 4).

Strand transfer is measured by the integration of an 18- and 20-bp recessed M-MuLV LTR DNA substrate (Fig. 1, step 2). The 18-nt strand, which has the conserved CA at the 3′ end, is 5′ ^{32}P labeled; autointegration results in products of variable sizes, depending on the site of integration, that are larger or smaller than the starting 18-nt substrate strand. Figure 3B, lane 2, shows the strand transfer activity for untreated M-MuLV IN. M-MuLV IN treated with DTT prior to NEM addition was only slightly reduced (Fig. 3B, lane 3). With M-MuLV IN preincubated with NEM, there was complete loss of strand transfer activity (Fig. 3B, lane 4). Strand transfer activity was also eliminated with M-MuLV IN modified with iodoacetate (data not shown). Therefore, both enzymatic activities of the forward integration reaction of M-MuLV IN were dependent on the availability of the Cys residues.

Catalysis mediated by NEM-modified M-MuLV integrase: effect on the joining activity of disintegration. The involvement of the Cys residues in the disintegration reaction was analyzed by assaying for the joining activity of disintegration. The Y substrate used to detect joining activity was Y3403, in which the C strand is ^{32}P labeled at the 5′ end (Fig. 1, step 4; Fig. 4, lane 1). A 30-bp product is generated by IN by joining the 5′ end of the cleaved B strand to the 3′ end of the ^{32}P-labeled C strand (Fig. 4, lane 5). Additional product is located above the 30-nt band shown in Fig. 4, which is due to secondary structure (6).

Preincubation of M-MuLV IN with either 20 mM NEM (Fig. 4, lane 2) or 10 mM NEM (Fig. 4, lane 3) resulted in 43 and 27% decreases in activity, respectively, with respect to the standard joining activity (Fig. 4, lane 5). While NEM eliminated the activities associated with the forward reaction, strand transfer activity, and cleavage activity (Fig. 3),

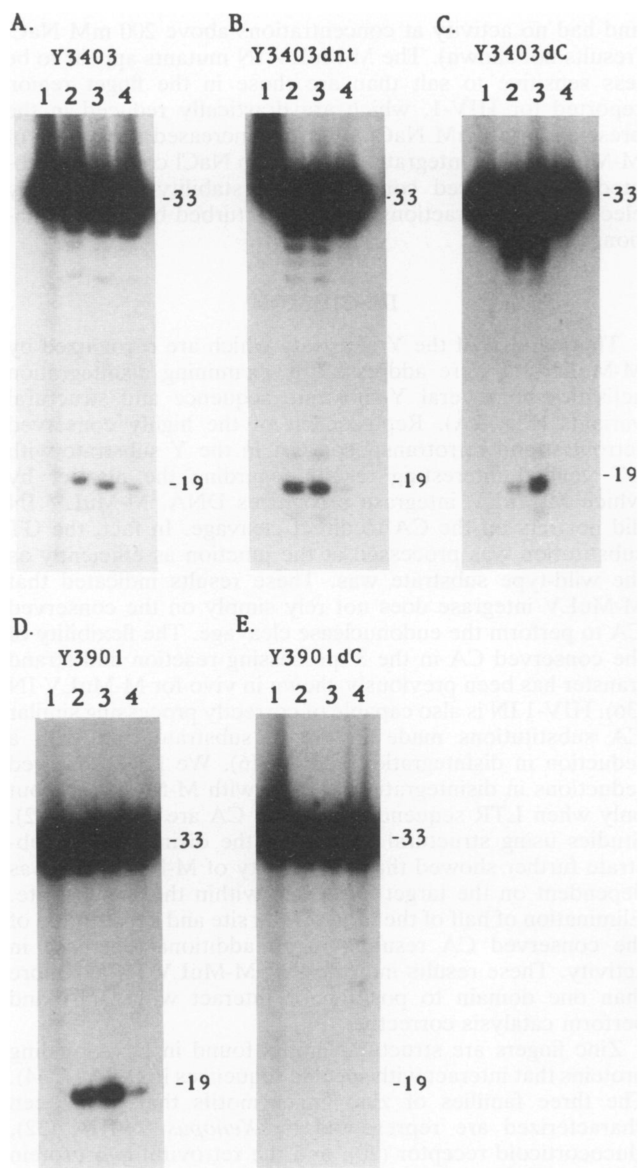


FIG. 5. Recognition of Y structural variants with NEM-treated M-MuLV IN and effects on endonucleolytic cleavage activities. M-MuLV IN was preincubated on ice as in previous experiments with either NEM or DTT and assayed for activity for 1 h with the substrates illustrated in panel A under conditions described in Materials and Methods following preincubation for 30 min. Reaction mixtures contained 0.2 pmol each of substrates Y3403 (A), Y3403dnt (B), Y3403dC (C), Y3901 (D), and Y3901dC (E) and 5 pmol of M-MuLV IN. Four reactions are shown for each substrate: lane 1, substrate and buffer; lane 2, IN preincubated on ice; lane 3, IN preincubated on ice with DTT; and lane 4, IN preincubated on ice with NEM. Products were separated on 20% sequencing gels, dried, and subjected to autoradiography and PhosphorImager analysis.

the joining activity of the reverse reaction was only slightly diminished by NEM (Fig. 4). Interestingly, preincubation with DTT as a control (Fig. 4, lane 4) resulted in a 1.5-fold enhancement of activity. This increase was observed only in assays for disintegration activity, not those for integration activity. This enhancement in activity of the reduced integrase may be attributed to a change to a preferred conformation, decrease in the multimerization state of M-MuLV IN, or minimization of protein oxidation.

Endonuclease activity mediated by NEM-modified M-MuLV integrase with use of the disintegration substrate and analogs. The results presented in Fig. 2 indicated a differential recognition by M-MuLV IN of several disintegration substrates. To determine whether the His-Cys region was involved in the observed differential recognition, the Cys residues of M-MuLV IN were modified with NEM and assayed for the endonuclease activity of disintegration with the various Y substrates, ^{32}P labeled on the B strand (Fig. 2A).

Reactions with the wild-type substrate, Y3403, are presented in Fig. 5A, lane 2. A slight decrease in the endonuclease activity was noted with M-MuLV IN modified with NEM (Fig. 5A, lane 4), similar to the effects observed for joining activity of disintegration (Fig. 4). In three separate experiments, the average activity of NEM-modified M-MuLV IN was 68% ± 5% of that of wild-type or untreated M-MuLV IN. The activity of NEM-modified M-MuLV IN was repeatedly found to be 4- to 9.6-fold lower with Y3901 than with Y3403. In this representative experiment, endonuclease activity of NEM-treated M-MuLV IN on Y3901 (Fig. 5D, lane 4) had 11% of the activity observed for the standard reaction with M-MuLV IN and Y3901 (Fig. 5D, lane 2). The NEM-modified M-MuLV IN was more vulnerable to change in the conserved CA, similar to the synergistic effects seen with the structural alterations of the target site within disintegration substrates.

The Y-substrate analogs were also examined with NEM-modified M-MuLV IN (Fig. 2A). The structural variants, Y3403dnt (Fig. 5B, lane 4) and Y3403dC (Fig. 5C, lane 4) yielded significant decreases to 12 and 16%, respectively, when incubated with NEM-modified M-MuLV IN. These results imply that the His-Cys finger region is not involved in target or host DNA recognition but is possibly a major domain for recognition or interaction of the M-MuLV LTR. Disintegration activity, as determined by the endonuclease assay for Y3901dC (Fig. 5E) and Y3901dnt (data not shown), was not detected with NEM-modified M-MuLV IN. As observed in Fig. 4, preincubation of M-MuLV IN under reducing conditions stimulated disintegration activity and demonstrated an even higher stimulation of activity with the Y3403dC substrate. This stimulation in activity was not seen with any of the Y3901 substrates.

Enzymatic activity of M-MuLV integrase with amino acid changes in the His-Cys region. Two mutant M-MuLV integrase proteins were examined for their integration and disintegration activities. Both of these mutations are in the His-Cys finger region and are lethal in vivo but do retain nonspecific DNA binding activity in vitro (37). One mutation is a linker insertion between amino acid residues S-63 and F-64 (*in*5247-12) adjacent to the second His in the M-MuLV IN finger region. The second mutation is a single amino acid change, C95Y (*bi*5349-1), of the latter Cys in the finger region. The mutant proteins were expressed in *E. coli* and purified similarly to the wild type.

The two mutations made in the His-Cys region were assayed for integration and disintegration activities. *in*5247-12 and *bi*5349 were inactive for integration (data not shown). The linker insertion (*in*5247-12) and C95Y (*bi*5349) mutants were assayed for the joining activity of disintegration. Both proteins (*in*5247 [Fig. 6A] and *bi*5349 [results not shown]) had approximately 10% of the wild-type M-MuLV IN disintegration activity. Initially this was a surprising result, since these mutations affect the same residues or area

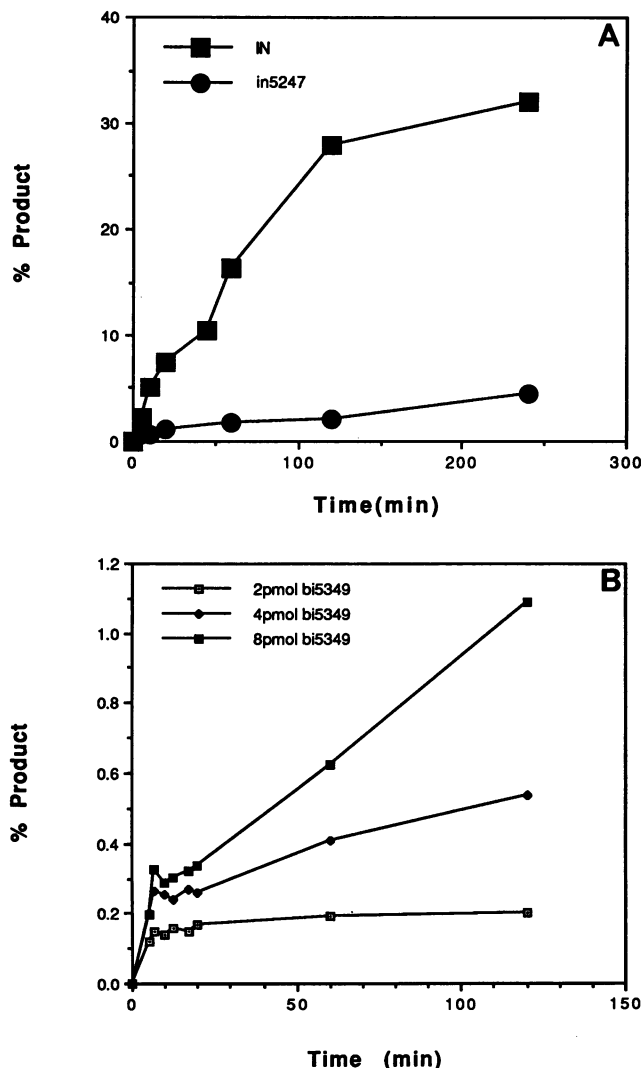


FIG. 6. Time course of disintegration with wild-type and mutant M-MuLV IN. (A) Disintegration activity was monitored for several hours for M-MuLV IN (4 pmol) and *in*5247-12 (4 pmol), using Y3403 (0.2 pmol) as a substrate. (B) Disintegration activity is presented for several concentrations of *bi*5349, using Y3403 (0.2 pmol) as a substrate. Percent product was determined by PhosphorImager analysis.

targeted by the NEM experiments. A time course of disintegration activity spanning 2 h for *bi*5349 is presented in Fig. 6B. Disintegration activity observed in both mutant enzymes did not demonstrate the kinetics typically noted with the wild-type M-MuLV IN upon incubation with disintegration substrates. Instead, disintegration activity showed an initial small burst which then increased steadily over 2 to 4 h. The rate of increase was dependent upon the concentration of M-MuLV IN in the reaction.

Previous biochemical characterization of M-MuLV integrase found the disintegration reaction to be highly tolerant to salt concentrations up to 0.5 M NaCl, in contrast to integration activity, which is salt sensitive (23). Characterization of the disintegration activity of *in*5247-12 and *bi*5349-1 revealed that the mutant proteins were greater than 70% decreased in activity in the presence of 100 mM NaCl and had no activity at concentrations above 200 mM NaCl (results not shown). The M-MuLV IN mutants appear to be less sensitive to salt than are those in the finger region reported for HIV-1, which are drastically reduced in the presence of 50 mM NaCl (47). The increased sensitivity of M-MuLV IN disintegration activity to NaCl could be attributed to a reduced conformational stability provided by electrostatic interactions that are perturbed by these mutations.

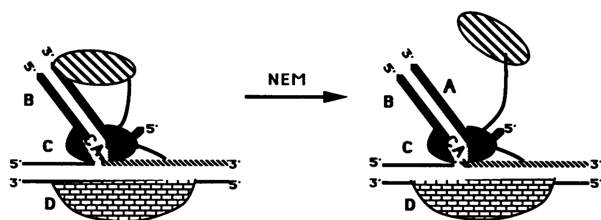
DISCUSSION

The features of the Y substrate which are recognized by M-MuLV IN were addressed by examining disintegration activities on several Y-substrate sequence and structural variants (Fig. 2A). Replacement of the highly conserved retroviral and retrotransposon CA in the Y substrate with GT yielded interesting results regarding the manner by which M-MuLV integrase recognizes DNA. M-MuLV IN did not rely on the CA to direct cleavage. In fact, the GT substitution was processed at the junction as efficiently as the wild-type substrate was. These results indicated that M-MuLV integrase does not rely simply on the conserved CA to perform the endonuclease cleavage. The flexibility of the conserved CA in the 3′-processing reaction and strand transfer has been previously shown in vivo for M-MuLV IN (36). HIV-1 IN is also capable of correctly processing similar CA substitutions made in the Y substrate, but with a reduction in disintegration activity (6). We have observed reductions in disintegration activity with M-MuLV IN, but only when LTR sequences 5′ to the CA are removed (12). Studies using structural analogs of the disintegration substrate further showed that the activity of M-MuLV IN was dependent on the target DNA site within the Y substrate. Elimination of half of the target DNA site and substitution of the conserved CA resulted in an additional decrease in activity. These results indicate that M-MuLV IN has more than one domain to position or interact with DNA and perform catalysis correctly.

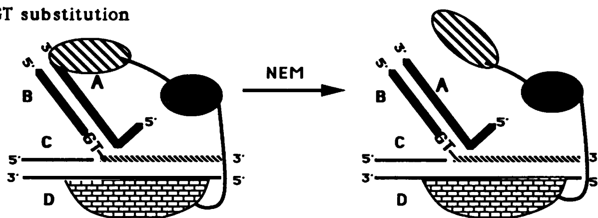
Zinc fingers are structural motifs found in DNA-binding proteins that interact with specific sequences in DNA (7, 44). The three families of zinc finger motifs that have been characterized are represented by *Xenopus* TFIIIA (32), glucocorticoid receptor (20), and the retroviral *gag* protein (45). Structural studies of these families of zinc finger proteins show that each family is distinct in terms of structure, DNA binding and recognition mechanisms, and protein-protein interactions (20). Although none of these families resemble the zinc finger motif conserved in all retroviral and retrotransposon IN proteins (21), by analogy, the involvement of the zinc finger motif in M-MuLV integrase-DNA interactions should be likely.

We have addressed the role of the putative zinc finger in M-MuLV IN by chemical modification and mutations. Chemical modification by NEM of the cysteine residues in M-MuLV IN revealed a difference in the importance of the finger region during integration and disintegration. We observed a loss of the 3′-processing and strand transfer activities of integration and a decrease but not a loss of activity in the endonucleolytic cleavage and joining activities of disintegration. The effects of the NEM modifications are attributed to the two Cys residues in the finger region and not the third Cys in the central core, as mutation of this third Cys residue to Ala resulted in wild-type levels of integration and disintegration activity (24). The apparent biphasic kinetics in disintegration activity of the His-Cys mutants may indicate

A. Standard Y-substrate



B. GT substitution



C. 3' Processing and strand transfer substrate

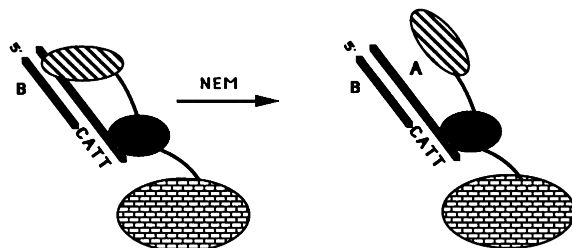


FIG. 7. Model of the disintegration and integration activities mediated by M-MuLV IN. Only one promoter is shown for simplicity of illustration. In addition, the domains are presented as separate, but this is not meant to imply that they are physically distinct. Three domains are proposed to be involved in substrate recognition. Domain 1 ( ) represents the zinc finger region primarily responsible for positioning the LTRs for strand transfer. Domain 2 ( ) represents the CA proximal site. Domain 3 ( ) is the region that mediates nonspecific DNA binding and is proposed to be involved in the structural recognition of the Y substrate.

that the finger mutations have affected the ability of M-MuLV IN promoters to interact with each other or with the substrate, presumably as a result of improper folding of this region. The His-Cys region of HIV IN is highly ordered (4), and mutation of the amino acid residues required for the secondary structure renders this region disordered. This finding helps to explain results with NEM modification of wild-type M-MuLV IN, which is presumably already in the correct conformation and would be less affected by chemical modification of the Cys residues. Thus, the presence of a misfolded domain may have effects not seen with the correctly folded but chemically modified proteins or proteins lacking the domain completely.

Based on our results, a working model for the interaction of M-MuLV IN and NEM-modified M-MuLV IN with the viral substrates in integration and disintegration is presented (Fig. 7). In Fig. 7, a single promoter is presented only for simplicity of illustration. The active protein oligomer for integration and disintegration is not known, although integrase is able to form a dimer or tetramer (19, 22, 47). The number of catalytic sites is also not known. However, every mutation in the proposed active site that blocks disintegration also affects integration, implying similar amino acids for catalysis (14, 15, 18, 31, 33, 47). In this model, the mode of recognition mediated by M-MuLV IN of viral and nonspecific DNA is distinct between the integration (Fig. 7C) and disintegration (Fig. 7A and B) reactions.

Our results suggest that a minimum of three domains in M-MuLV IN are involved in substrate recognition. The interactions of these three domains for the three categories of substrates examined are illustrated as separate entities for clarity only, not to imply a lack of domain interaction or location. Domain 1 represents the zinc finger region. The 3'-processing and strand transfer activities may require contact with the putative zinc finger (domain 1) or a conformation provided by this region (Fig. 7C). The zinc finger domain is not involved in sequence-specific DNA binding or strong nonspecific DNA binding (27, 34, 40, 51) but is required for integration activity of IN, as demonstrated herein for M-MuLV IN as well as elsewhere (14, 18, 47) for HIV IN. Therefore, we favor a model in which the finger region may be involved in positioning of the LTR and/or providing a three-dimensional structure required for integration activity. This three-dimensional structure may provide a pocket for positioning of the viral and target DNA. From our studies with the M-MuLV IN mutants and NEM-modified M-MuLV IN, we speculate that the disintegration reaction does not rely on interaction with this domain. Interestingly, mutations in the zinc finger were impaired in disintegration activity more than the NEM-modified M-MuLV IN was. This difference may be attributed to the inability of the mutant protomers to refold properly and assume the correct three-dimensional conformation or multimerization. The wild-type M-MuLV IN is presumably already in the conformation needed for optimal enzymatic activity, and treatment with NEM may not affect multimerization of the protein. Analysis of the kinetics of disintegration for the His and Cys mutants further demonstrated this possible cooperativity between promoters.

The results of the dinucleotide substitution of the conserved CA with a GT in the Y substrate suggests a functional domain proximal to the CA or Y junction (domain 2 in Fig. 7B). In the case of integration, domains 1 and 2 would both be required for activity. Interestingly, domain 2 appears to be more central to optimal activity in HIV-1 IN than in MuLV IN as well as more flexible in terms of disintegration (6, 12, 41, 47). In contrast to M-MuLV IN, the disintegration activity of HIV-1 IN is greatly decreased with substitution of the conserved CA in the Y substrate. M-MuLV IN was also unable to perform any detectable catalysis with the variety of substrates demonstrated to have the splicing activity with HIV-1 (12). For M-MuLV IN, our results suggest that domain 1 was more important for optimal activity than was found for HIV-1 IN.

Finally, a third domain is proposed as a region in M-MuLV IN that mediates nonspecific DNA binding. M-MuLV IN has nonspecific DNA binding properties that are not sensitive to NEM (38). Therefore, the third domain is proposed to be involved in recognition of the target DNA region and structure of the disintegration substrate. In terms of integration in vivo, this region may then mediate the nonspecific binding to the host DNA that is critical for integration. Domain 3 may be attributed to the carboxyl half of IN, which has been implicated as mediating nonspecific DNA binding affinity in studies of several retroviral integrases (27, 34, 40, 51).

The mechanism by which the finger regions of the retro-

viral integrases may recognize or position the viral LTR is biochemically intriguing. There are several possibilities, such as hydrophobic interaction with the methyl groups in the thymine-rich region of the M-MuLV LTR. In M-MuLV IN, 53% of the residues that comprise the zinc finger containing the two His residues are hydrophobic. In HIV-1 IN, the region between the His and Cys residues is composed of 52% hydrophobic residues. The influence of this region on the folding of the protein may also be of importance. Integration by M-MuLV IN is extremely sensitive to the ionic strength of the reaction environment, indicating that the His-Cys region or another part of IN could potentiate DNA binding through electrostatic interactions (23). Likewise, the His-Cys finger may rely on electrostatic interactions with another part of IN or protomer to assume a particular conformation that is competent for integration. Furthermore, mutations in the finger region also conferred an increased sensitivity to the ionic strength in disintegration reactions.

Endonuclease activity of M-MuLV IN was determined for several structural derivatives of the disintegration substrate. The target DNA as well as the viral LTR portions of the disintegration substrate were determined to be important for substrate recognition and catalysis by M-MuLV IN. However, M-MuLV IN appears to have a greater requirement for sequences upstream of the LTR than HIV-1 IN does (6). This requirement was also observed in studies that showed that a progressive elimination of the LTR within the Y structure resulted in a progressive loss of disintegration activity with M-MuLV IN (12). Indeed, M-MuLV IN and HIV-1 IN have similar enzymatic properties, but the substrate requirements of each retrovirus are unique as well as complex. This is readily apparent in the conservation of the LTR sequences, which are perfectly conserved for 13 bp in M-MuLV, while only the terminal 4 bp are conserved in HIV-1. Studies with M-MuLV in vivo show that the terminal 12 bp of the LTR are required for integration (8, 9, 35, 36), and in vitro studies have shown that the terminal 9 bp will provide 77% of the wild-type levels of integration (5, 49). Interestingly, M-MuLV IN is able to integrate an HIV LTR in vitro (5), but HIV-1 IN is not able to process or catalyze a strand transfer reaction with an oligonucleotide which mimics the M-MuLV LTR (30, 41, 48). Certainly, an understanding of the differences as well as similarities exhibited by retroviral integrases will provide insight into the biochemistry of this family of proteins.

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