Modulation of Activity of Moloney Murine Leukemia Virus Preintegration Complexes by Host Factors In Vitro

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We have explored the requirements for host proteins in the integration of Moloney murine leukemia virus (MoMuLV) cDNA in vitro. Following infection, it is possible to lyse cells and obtain preintegration complexes (PICs) capable of integrating the MoMuLV cDNA into an added target DNA in vitro (intermolecular integration). PICs can be stripped of required proteins by gel filtration in high-salt buffers (600 mM KCl), allowing the nature of the removed factors to be investigated by in vitro reconstitution. In a previous study of human immunodeficiency virus type 1 (HIV-1) PICs, the host protein HMG I(Y) was found to be able to restore activity to salt-stripped PICs. In contrast, salt stripping and reconstitution of MoMuLV PICs led to the proposal that a host factor is important for a different activity, blocking integration into the cDNA itself (autointegration). In this report, we investigated reconstitution of salt-stripped MoMuLV PICs and found that addition of cellular extract from uninfected NIH 3T3 cells could block autointegration and also restore intermolecular integration. Isolation of the intermolecular integration-complementing activity yielded HMG I(Y), as in the HIV-1 case. However, HMG I(Y) could not block autointegration, implicating a different host factor in this process. Additionally, when MoMuLV PICs were partially purified but not salt stripped, the intermolecular integration activity was reduced but could be stimulated by the addition of any of several purified DNA binding proteins. In summary, three activities were detected: (i) the intermolecular integration cofactor HMG I(Y), (ii) an autointegration barrier protein, and (iii) stimulatory DNA binding proteins.

An essential step in retroviral replication is the integration of viral cDNA into host cell DNA (11, 20, 54). In vivo, the linear viral cDNA is assembled into large nucleoprotein complexes called preintegration complexes (PICs) (2, 26, 44). PICs can be isolated from newly infected cells and are capable of integrating the viral cDNA into an added target DNA in vitro (3, 16, 21). We have used such PIC-based assays to investigate host factors important for integration of Moloney murine leukemia virus (MoMuLV) cDNA.

One of the components of MoMuLV PICs is inferred to be integrase. Though the protein has not yet been detected physically in isolated MoMuLV PICs, integrase is expected to be present, since the enzymatic activity is essential for integration in vivo (4, 14, 50). For human immunodeficiency virus type 1 (HIV-1), integrase has been shown to be associated with PICs (5, 18, 23). Recombinant integrase proteins from ASLV (avian sarcoma-leukosis virus), MoMuLV, HIV-1, and other retroviruses have been shown to be capable of removing 2 nucleotides from 3′ ends of model long terminal repeat (LTR) DNAs (12, 30, 38, 51) and joining the resulting recessed 3′ hydroxyl group to the target DNA in vitro (8, 12, 15, 35, 37).

Purified integrase proteins differ in their abilities to carry out coordinated joining of pairs of LTR ends, thereby generating integration intermediates with a defined spacing between points of joining at each end of the cDNA (called coupled joining) (Fig 1). In the case of HIV-1 integrase, only single LTR analogs generally become joined to target DNA, mimicking events at one cDNA end only (7, 8). More-efficient coupled joining is seen when lysed HIV-1 virions are used as a source of integrase (29). Purified MoMuLV and ASLV integrases yield coupled products more efficiently than does integrase of HIV (1, 12, 24, 37, 55).

Unlike purified integrase, reactions with PICs yield coupled integration products as the only detectable forms, producing the integration intermediate (II) illustrated in Fig. 1 (3, 16, 21). Integration is thought to be completed in vivo by host DNA repair enzymes which connect the remaining unjoined strands in the integration intermediate and complete proviral synthesis.

Several previous studies have outlined the organization and composition of PICs. In the case of MoMuLV, PICs are known to be large particles (around 160S) potentially containing several viral proteins (2). In the case of HIV-1, the linear viral cDNA is packaged into a large particle with a diameter of 54 nm as estimated by gel filtration (for comparison, the diameter of a virion is about 120 nm) (44). The ends of the cDNA in PICs are protected from exonuclease digestion by bound proteins (44). The two ends of viral cDNA appear to be held together by a protein bridge, since the cDNA can be cleaved internally without disrupting coordinated integration of the two cDNA ends (44). Of the viral proteins, integrase, reverse transcriptase (RT), matrix (MA) and Vpr proteins have been proposed to be the components of PICs and NC protein has been detected in some studies (5, 23, 28, 32, 44). In addition, a host protein, HMG I(Y) was found to be present in HIV-1 PICs and required for function in vitro (18). In that study, a salt-stripping procedure was used to remove a subset of proteins from HIV-1 PICs, which abolished intermolecular integration activity. Human HMG I(Y) protein was found to re-store the activity (18).

A salt-stripping method was used previously to identify a
were grown in Dulbecco modified Eagle's medium with 10% fetal calf serum and 2% protein per ml was frozen in liquid nitrogen and stored at -70°C. All the cells were grown in Dulbecco modified Eagle's medium with 10% fetal calf serum and 2% protein per ml was frozen in liquid nitrogen and stored at -70°C.

Extract containing the PICs used in Fig. 7 was prepared by a modification of the method described in reference 13. Newly infected cells were harvested by trypsinization and washed with ice-cold phosphate-buffered saline with 5 mM MgCl2. Cells were then incubated with 4 packed cell volumes of buffer B (10 mM Tris- HCl [pH 7.9], 10 mM KCl, 1.5 mM MgCl2, 1 mM dithiothreitol) on ice for 20 min and homogenized with 20 strokes (with a B pestle) in a Dounce homogenizer at 4°C. The cell lysate was clarified by low-speed centrifugation (1,000 x g, 3 min). The supernatant containing cytoplasmic PICs was further clarified by high-speed centrifugation (8,000 x g, 3 min), and the salt concentration was adjusted to 150 mM. The supernatant containing cytoplasmic PICs was frozen in liquid nitrogen and stored at -70°C.

Salt-stripping of PICs. One milliliter of crude PICs was partially purified by low-ionic-strength precipitation as described previously (19), except that 8,000 x g instead of 2,000 x g was used to pellet PICs. After low-ionic-strength precipitation, 80 to 90% of PICs could be recovered. After the PIC pellet was suspended in buffer A, 30% of the PICs could carry out intermolecular integration and no autointegration was detected. The protein concentration of the partially purified PICs was ~0.18 mg/ml and the concentration of viral DNA was ~8 ng/ml.

To carry out high salt depletion, the pellet PICs were suspended in 680 μl of buffer A with 600 mM KCl and incubated at room temperature for 10 min. The PICs were then subjected to gel filtration through Sepharose CL-4B spin columns equilibrated in buffer A with 600 mM KCl. The resulting salt-stripped PICs were concentrated by a Centricon-100 to a final volume of 50 to 100 μl. The concentrated salt-stripped PICs were then diluted to the desired volume with buffer A containing 600 mM KCl. Upon dilution to 150 mM KCl, the salt-stripped PICs lacked intermolecular integration activity and 10 to 15% of the PICs had autointegration activity.

Integration assay. The integration assay was carried out in buffer A with 30% glycerol and 10 μg of linearized φX174 RFI per ml. The reaction mixture was incubated at 37°C for 30 min. In the assay for complementing activity, the cellular extract or purified protein was incubated with salt-stripped PICs at room temperature for 10 min. The reaction buffer was then adjusted to the condition described above and further incubated at room temperature for 10 min. Linearized φX174 RFI was added last, and the reaction mixture was incubated at 37°C for 30 min.

PICs containing about 500 pg of viral cDNA were used in each reaction mixture. The total reaction volume was adjusted to 110 μl in all experiments. The DNA was purified and analyzed by Southern blotting with a 32P-labeled probe containing an LTR sequence. The intermolecular integration and autointegration activities were quantitated by PhosphorImager analysis (see Fig. 3A and 7).

Materials. Proteins were obtained from the following sources: Escherichia coli single-stranded DNA binding protein (SSB), Stratagene; T4 gene 32 protein, Boehringer Mannheim; and RNase A, Qiagen. HIV-1MN p7 was obtained from A. Dusty Miller. The plasmid was transfected into NIH 3T3 cells and MoMuLV was harvested 2 days later. Fresh NIH 3T3 cells were infected by the virus and passaged to establish a constantly infected cell line. The infected cells (2.4 x 10^6 cells) were plated together with uninfected NIH 3T3 cells (9.6 x 10^6) in a 150-mm-diameter dish. Cells were harvested after 16 h of coculture by trypsinization. The trypsinized cells were washed twice with buffer A (20 mM HEPES-NaOH [pH 7.4], 150 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol) and then lysed with 450 μl of buffer A containing 0.025% digitonin (Calbiochem) and 2 μg of aprotinin (Calbiochem) per ml. The lysate containing PICs was centrifuged at 150,000 x g for 20 min and homogenized with 20 strokes (with a B pestle) in a Dounce homogenizer on ice for 20 min and homogenized with 20 strokes (with a B pestle) in a Dounce homogenizer at 4°C. The cell lysate was clarified by low-speed centrifugation (1,000 x g, 3 min). The supernatant containing cytoplasmic PICs was further clarified by high-speed centrifugation (8,000 x g, 3 min), and the salt concentration was adjusted to 150 mM. The supernatant containing cytoplasmic PICs was frozen in liquid nitrogen and stored at -70°C.

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Here we have isolated a factor that stimulates intermolecular integration by salt-stripped MoMuLV PICs and found the factor to be murine HMG I(Y). We have determined that murine HMG I(Y) is not itself the autointegration barrier factor. Furthermore, we investigated stimulation by additional DNA binding proteins in different experimental settings. These data identify several activities important for function of MoMuLV PICs in vitro.

FIG. 1. Inferred pathway for intermolecular integration and autointegration. MoMuLV cDNA (thin lines) and target DNA (thick lines) are indicated. The 5' ends (small solid circles) and 3' ends (arrows) are indicated. II, integration intermediate.
RESULTS

Depletion of required factors from MoMuLV PICs with high concentrations of salt. To investigate host activities involved in integration, a salt-stripping protocol was tested to determine whether it removed factors required for integration by MoMuLV PICs, as had previously been observed for HIV-1 PICs (18). MoMuLV PICs were prepared from newly infected cells as described previously (3, 27) and partially purified by precipitation in the presence of low-ionic-strength buffers (75 mM KCl). Thirty percent of the partially purified PICs could integrate their viral cDNA into added φX174 target DNA, and no autointegration activity was detected (Fig. 2B, lane 2; see Fig. 3A, lane 1, also). To deplete the partially purified PICs of protein factors, low-salt-precipitated PICs were suspended in high-salt buffer (600 mM KCl) and subjected to gel filtration through Sepharose CL-4B in 600 mM KCl. Complexes such as PICs with a molecular mass greater than 2 × 10^7 Da passed through the column but free proteins were retained.

As previously reported (41), the resulting salt-stripped PICs had autointegration activity, as indicated by the formation of a circular form of the viral cDNA, but lacked intermolecular integration activity, as indicated by a disappearance of form II (Fig. 2B, lane 4). Note that the production of the circular autointegration product was independent of the presence or absence of added φX174 target DNA (Fig. 2B, compare lanes 3 and 4). These results indicated that the salt-stripping removed factors required both for the protection of viral cDNA from autointegration and for efficient intermolecular integration.

Analysis of reaction products by cleavage with restriction enzymes. To verify that the DNA products were those illustrated in Fig. 1, the structures of the reaction products were probed by cleavage with restriction enzymes (Fig. 2C and D). HindIII cleaves MoMuLV cDNA once but does not cut the φX174 target DNA. BamHI cleaves MoMuLV cDNA three times but does not cleave the target. Reaction products made with (Fig. 2, lanes 2 and 4) or without target DNA (Fig. 2, lanes 1 and 3) were digested with HindIII (Fig. 2C) or BamHI (Fig. 2D). The sizes of fragments expected to be detected by the LTR probe are shown in Fig. 2A, and sizes detected on Southern blots are shown beside the autoradiograms. Note that the expected autointegration product described is the inverted-circle form. A second autointegration product, pairs of DNA circles, are not detected by this method (see Materials and Methods and reference 22 for details). All the DNA sizes observed in Fig. 2B to D are as expected by the model in Fig. 1 and Fig. 2A, indicating that the observed DNA bands are likely the expected intermolecular integration and autointegration products. A similar analysis of MoMuLV autointegration by cleavage with BamHI was published previously (41).

Reconstitution of salt-stripped PICs with host cell extract. The nature of the factor(s) required to restore intermolecular integration and block autointegration was then investigated. Cytoplasmic or nuclear extract from NIH 3T3 cells was used to reconstitute salt-stripped PICs (Fig. 3A, lanes 3 to 8). Each extract was able to restore intermolecular integration (indicated by the arrow labeled II) and showed some ability to block autointegration.
used to complement salt-stripped PICs. The nuclear extract depleted by cellulose could complement intramolecular integration (Fig. 3B, lane 3). However, extract depleted by single- or double-stranded DNA-cellulose failed to complement (Fig. 3B, lanes 4 and 5). Evidently the complementing activity is contributed at least in part by a DNA binding protein, particularly one that can bind both single- and double-stranded DNA.

Analysis of the target of the complementing activity by order-of-addition experiments. To investigate whether the complementing factor acts on the salt-stripped PIC or the target DNA, an order-of-addition experiment was performed. The salt-stripped PICs were incubated with nuclear extract either before or after addition of target DNA. Note that the dX174 target DNA was present in great excess over viral DNA (10 μg/ml versus 8 ng/ml), so the dX174 DNA can act as a sink for added DNA-binding factors as well as an integration target. Premixing target DNA with complementing nuclear extract substantially reduced intermolecular integration upon addition of salt-stripped PICs (Fig. 4, compare lanes 2 to 4 with lanes 5 to 7). These findings support a model in which the complementing activity acts on PICs and not on target DNA.

Fractionation of HMG proteins in NIH 3T3 extract identified HMG I(Y) as the intermolecular integration-complementing factor. Since the factor restoring activity to salt-stripped PICs or the target DNA, an order-of-addition experiment was performed. The salt-stripped PICs were incubated with nuclear extract either before or after addition of target DNA. Note that the dX174 target DNA was present in great excess over viral DNA (10 μg/ml versus 8 ng/ml), so the dX174 DNA can act as a sink for added DNA-binding factors as well as an integration target. Premixing target DNA with complementing nuclear extract substantially reduced intermolecular integration upon addition of salt-stripped PICs (Fig. 4, compare lanes 2 to 4 with lanes 5 to 7). These findings support a model in which the complementing activity acts on PICs and not on target DNA.

Fractionation of HMG proteins in NIH 3T3 extract identified HMG I(Y) as the intermolecular integration-complementing factor. Since the factor restoring activity to salt-stripped PICs of HIV-1 was also a DNA binding protein and proved to be an HMG protein (18), HMG proteins were tested for the ability to complement salt-stripped MuLV PICs. Total HMG proteins were extracted from NIH 3T3 cell nuclei and loaded onto a Mono-S ion-exchange column. The column was eluted with a NaCl concentration gradient. Fractions were analyzed for complementing activity (Fig. 5A) and for protein content by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by staining with Coomassie brilliant blue (Fig. 5B). Fractions 20 and 22 complemented intermolecular integration activity of salt-stripped PICs (Fig. 5A, lanes 10 and 11).

Proteins in fractions 20 and 22 had the mobility on SDS-polyacrylamide gels and the fractionation profile on the Mono-S ion-exchange column expected for HMG I(Y) (18). Western blot analysis using an anti-HMG I(Y) antibody established that these fractions contained HMG I(Y) (Fig. 5C).

In addition to fractions 20 and 22, fraction 24 also contained HMG I(Y) but lacked complementing activity (Fig. 5A, lane 11, and Fig. 5C, lane 12). However, this fraction also contained histone H1 (Fig. 5B, lanes 12 to 14, band at about 30 kDa) which was previously found to inhibit intermolecular integration (18, 18a). Histone H1 also inhibited autointegration (Fig. 5A, lanes 11 to 13). Therefore, with the exception of the fractions which contained inhibitory histone H1, all the fractions containing HMG I(Y) were able to complement intermolecular integration by salt-stripped PICs.

As described above, the intermolecular integration-complementing activity was present in both cytoplasmic and nuclear fractions, as is HMG I(Y) (18). The relative amounts of activity in each did not correlate with the abundance of HMG I(Y) in a simple way since each fraction is a mixture of stimulatory HMG I(Y) and inhibitory factors such as histone H1 (unpublished data). In addition, the complementing activity could bind to both single- and double-stranded DNA-cellulose, as is known to be the case for HMG I(Y) (18). These observations further support the attribution of the intermolecular integration-complementing activity to HMG I(Y).

Complementation with purified proteins. To confirm that HMG I(Y) was the intermolecular integration-complementing activity, recombinant HMG I(Y) protein was purified after overexpression in bacteria and tested. In this experiment, HMG I(Y) was attached to a hexahistidine tag for convenient purification [named (HT)HMG I(Y)]. In the presence of the purified (HT)HMG I(Y), the intermolecular integration activity of salt-stripped PICs was partially restored (Fig. 6, lanes 2 to 4). However, (HT)HMG I(Y) had consistently lower complementing activity than uninfected-cell nuclear extract.
At all concentrations tested, none of these DNA binding proteins displayed complementing activity. Addition of bovine serum albumin did not stimulate the reaction and at the highest concentration, the integration activity was increased by 1.5- to twofold (Fig. 7, lanes 13 to 20).

A separate requirement for blocking autointegration. Several observations indicate that the activity that restores intermolecular integration is different from the previously reported factor (41) that blocks autointegration. (i) Although nuclear extract complemented salt-stripped PICs as well as cytoplasmic extract, nuclear extract had much higher autointegration blocking activity (2 μl of nuclear extract could reduce autointegration from 14% to 8%, while 2 μl of cytoplasmic extract had no detectable effect on autointegration [both extracts at 4 μg/μl]). These results suggested that different factors were responsible for each activity. (ii) DNA cellulose depleted nuclear extract complementing activity more efficiently than DNA cellulose depletion of nuclear extract complementing activity (data not shown) were also tested for complementing activity. At all concentrations tested, none of these DNA binding proteins displayed complementing activity.

FIG. 7. Stimulation of the intermolecular integration activity of partially purified but not salt-stripped PICs by purified DNA binding proteins. The PICs were purified by hypotonic lysis of cells followed by gel filtration through Sepharose CL-4B in 150 mM KCl. The PICs were then mixed with the purified protein at the concentrations indicated. All the reactions were performed under standard reaction conditions in a total volume of 110 μl BSA, bovine serum albumin.

Addition of bovine serum albumin did not stimulate the reaction and at the highest concentration, the integration activity was increased by 1.5- to twofold (Fig. 7, lanes 13 to 20). Addition of bovine serum albumin did not stimulate the reac-
tion (Fig. 7, lanes 21 to 24), indicating that simply increasing the bulk protein concentration did not account for the observed stimulation. Similar results were obtained (data not shown) when PICs were partially purified by a different method (digitonin lysis followed by low-ionic-strength precipitation and gel filtration through Sepharose CL-4B in 150 mM KCl). The observed diversity of DNA binding proteins that satisfy this requirement is in contrast to the strict requirement for HMG I(Y) in reconstituting intermolecular integration by salt-stripped PICs.

**DISCUSSION**

Here we report the isolation of a factor that restores intermolecular integration by salt-stripped MoMuLV PICs and its identification as murine HMG I(Y). A requirement for human HMG I(Y) was reported previously for HIV-1 PICs (18). The previously described autointegration barrier factor (41) was also detected in our experiments and found to be separate from HMG I(Y). We also identified another activity. When PICs were partially purified but were not salt stripped, they often had reduced intermolecular integration activity. This reduced activity permitted another approach to reconstitution, which revealed a requirement that could be satisfied by several different DNA binding proteins.

**Possible roles of HMG I(Y).** The mechanism by which HMG I(Y) complements the intermolecular integration by salt-stripped PICs is unclear, although several models can be proposed based on previous work. HMG I(Y) binds A/T-rich DNA sequences and such sequences are present in the LTRs of MoMuLV and HIV-1 (17, 42, 48, 49, 53). Each HMG I(Y) monomer has three DNA binding motifs (48) and one HMG I(Y) molecule can bridge two DNA's by binding to sites on each (33), leading to the possibility that HMG I(Y) acts by bringing distant DNA domains into proximity.

One possible model is that one HMG I(Y) molecule binds both viral cDNA and target DNA, thus bridging the PIC and target. However, a study of 61 integration sites generated by HIV-1 infection did not show an enrichment for HMG I(Y) binding sites near integration sites (10a), which is evidence against this model. Furthermore, the order-of-addition experiment in Fig. 4 also indicates that HMG I(Y) probably acts on PICs and not on target DNA.

An attractive model holds that HMG I(Y) may serve as an architectural element for the formation of active PICs, helping shape the DNA for optimal function. Host DNA binding proteins have been shown to serve as architectural elements in the assembly of recombination complexes in several well-studied site-specific recombination and transposition systems (31, 40, 45). In addition, HMG I(Y) itself has been proposed to serve as an architectural element in the assembly of transcription complexes (for a review see reference 9).

**HMG I(Y) protein is not the autointegration barrier activity.** Our data indicate that HMG I(Y) is not the autointegration barrier activity. Fractionation of murine HMG proteins yielded an activity that stimulated intermolecular integration by salt-stripped PICs, but no activity that redirected autointegration to intermolecular integration was seen. Purified HMG I(Y) could restore intermolecular integration activity of salt-stripped PICs but could not block autointegration. In addition, the relative amounts of the two activities differed in nuclear and cytoplasmic fractions of uninfected host cells, consistent with different factors accounting for each.

**Stimulation of partially purified PICs by diverse DNA binding proteins.** We found that MoMuLV PICs that had been partially purified but not salt stripped were often diminished in intermolecular integration activity, and the activity could be boosted two- to fourfold by addition of any of several DNA binding proteins. RNase A, T4 gene 32 protein, E. coli SSB, HMG I(Y), HIV-1 NC, and HMG-1 all supported this activity. This stimulation is distinct from the reconstitution of intermolecular integration activity of salt-stripped PICs, since only HMG I(Y) could reconstitute in this setting. Of the proteins that stimulated PICs in the two assays, so far only HMG I(Y) has been found in association with PICs (in the HIV-1 system [18]). Similar composition studies have not yet been possible in the MoMuLV system.

It is unclear how these diverse DNA binding proteins promote integration in vitro. Previously many of these proteins have been found to stimulate reactions with purified integrase proteins. Purified MoMuLV integrase was stimulated 3 to 13-fold by E. coli SSB, RNase A, and T4 gene 32 protein (12a), purified ASLV integrase was stimulated two- to fourfold by HMG-1 and HU proteins (1), and purified HIV-1 integrase was stimulated very strongly by NC protein (10), two- to fourfold by RNase T1 (43), 10 to 20-fold by Ini-1 (36), and strongly by a cocktail of DNA binding proteins (8). The fact that so many proteins support this activity raises the possibility that the mechanism involves nonselective coating of DNA, perhaps by shielding negative charges on the DNA phosphates (though for a different view, see reference 1). It will be of interest to determine whether this activity is important for integration in vivo and, if so, what proteins are involved.

**Possible further host activities important for the function of PICs.** HMG I(Y) purified after overexpression in E. coli was less active for complementation than extracts from uninfected NIH 3T3 cells, raising the possibility that still other factors are involved. Furthermore, in the HIV-1 case, purified HMG I(Y) also did not reconstitute activity as well as crude extracts did (18). The complementation experiments illustrated in Fig. 6 used human HMG I, not murine HMG I which differs from human HMG I at two amino acid positions (34). Therefore, it is also possible that this difference explains the discrepancy in activities. HMG I(Y) has been reported to be posttranslationally modified by glycosylation, phosphorylation, acetylation, methylation, and adenylation (46, 47). Possibly, the lack of posttranslational modifications in the purified HMG I(Y) accounts for the low complementing activity. Alternatively, further protein factors may be involved. The methods for stripping and reconstituting PICs described offer an approach to identifying possible additional factors.

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