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Protection of retroviral DNA from autointegration: Involvement of a cellular factor

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ABSTRACT **An essential step in the retrovirus life cycle is integration of a DNA copy of the viral genome into a host chromosome. After reverse transcription, there can be a delay of many hours before the viral DNA is integrated. It is important for the retrovirus to ensure that the viral DNA does not integrate into itself during this period; such autointegration is a suicidal process that would result in destruction of the viral genome. Understanding of the mechanism that blocks autointegration of the viral DNA may lead to insights into how to inhibit viral replication by inducing the viral DNA to autointegrate. Evidence is presented in this report that viral nucleoprotein complexes isolated from cells infected with Moloney murine leukemia virus exhibit a barrier to autointegration. The barrier can be disrupted by high salt treatment and, subsequently, restored by addition of factors provided by a host cell extract. Our data indicate an involvement of host machinery in protecting retroviral DNA from autointegration.**

An infecting retrovirus constitutes a package of enzymes and substrates required for the subsequent steps of reverse transcription and DNA integration (1–4). Thus, the retroviral DNA, synthesized by reverse transcription, forms part of a high molecular weight nucleoprotein complex derived from the core of the infecting virion. This integration-competent nucleoprotein complex (INC) (5–13) directs a defined set of DNA cutting and joining reactions (6, 7, 14) (Fig. 1) that insert the viral DNA into the host genome. The viral integrase protein must be present in the complex since it is required for the key enzymatic steps of the integration reaction (15–20), but the other protein components of the complex and their functional roles are yet to be determined.

Although purified integrase protein alone can catalyze a concerted DNA cleavage–ligation reaction *in vitro* (18–20), the authentic INCs manifest two properties that are not reproduced in reactions with purified integrase and synthetic DNA substrates. (*i*) The INCs efficiently insert both viral DNA ends into a target DNA in a pairwise manner (5–12); the major products of reactions with integrase result from half-reactions in which a single viral DNA end is inserted into a single strand of the duplex target DNA (18, 21). (*ii*) The INCs of both Moloney murine leukemia virus (MoMLV) (6) and human immunodeficiency virus type 1 (11) preferentially integrate the viral DNA intermolecularly into a target DNA, avoiding the intramolecular autointegration pathway. The molecular bases of these differences are yet to be explored.

What features of the INC are responsible for favoring the intermolecular integration reaction over autointegration? One possibility is that a physical barrier makes the viral DNA unavailable as a target for integration. To investigate this issue, we devised an assay to simultaneously analyze both autointegration and intermolecular reaction products generated *in vitro* with the INC isolated from MoMLV-infected cells (Fig. 2). Evidence is presented here that the INC of MoMLV indeed possesses a barrier to autointegration. The data also indicate that establishment of the autointegration barrier requires a trans-acting factor(s) of host cellular origin.

MATERIALS AND METHODS

Preparation of the INC. Cytoplasmic extract (fraction I) containing the INC was prepared, after coculture of NIH 3T3 fibroblasts and clone 4, a MoMLV-producing cell line (6), as described (5, 6) with the following modifications. (*i*) The extraction buffer was 20 mM Hepes·NaOH, pH 7.5/5 mM $MgCl_2$/150 mM KCl/10 mM dithiothreitol/0.002% (wt/vol) aprotinin (Sigma)/0.025% digitonin (Sigma). (*ii*) After removal of nuclei, 6 mM EDTA and 6% (wt/vol) sucrose were added to the cytoplasmic extract. Addition of EDTA prevented autointegration of the INC during further purification (data not shown). Fraction II was prepared by spin dialysis through a 0.8-ml column of Sepharose 4B-CL (Pharmacia) formed in a 1.5-ml Eppendorf tube and equilibrated with 20 mM Hepes·NaOH, pH 7.5/5 mM $MgCl_2$/6 mM EDTA/150 mM KCl/1 mM dithiothreitol/6% sucrose to remove relatively small molecules.

Integration Assay. Standard integration reaction mixtures (100–300 μl) contained 20 mM Hepes·NaOH (pH 7.5), 200 mM KCl, 10 mM $MnCl_2$, 10 mM dithiothreitol, 0.02% bovine serum albumin, 15% (wt/vol) glycerol, 3 nM φX174 replicative form I DNA as the target, and 15 amol (10^7 copies) of INC (5–10 μl of fraction II). $MgCl_2$ was substituted for $MnCl_2$ in some reactions; either of these divalent cations supports both intermolecular and autointegration reactions. The reactions were incubated at 37°C for 15 min, and products were deproteinized by incubation with proteinase K, extracted with a 1:1 mixture of $CHCl_3$/phenol followed by $CHCl_3$, and then ethanol-precipitated. As shown in Fig. 2, the reaction products were cleaved with *Bam*HI and then separated by agarose gel electrophoresis. After Southern blot transfer, the products were detected by hybridization with a ^{32}P-labeled probe containing MoMLV long terminal repeat (LTR) sequences (6) followed by exposure of Kodak XAR-5 film or a phosphor screen.

Quantitation of Integration Reaction Products. Products were quantitated with a PhosphorImager (Molecular Dynamics) by measuring the total density (integrated volume) of rectangles enclosing the entire gel lane (*X*), the 11.0-kb intermolecular product band (*Y*), and the 5.6-kb band derived from autointegration products (*Z*). The percentage of intermolecular integration products was calculated as $(Y/X) \times 100\%$. The corrected percentage of autointegration products was estimated to be $(3Z/X) \times 100\%$. The factor of 3 is introduced because if autointegration occurs at random locations in the target DNA and there is no bias for 3′-end joining to the same or opposite DNA strands, only one-third of the autointegration products would generate this band upon *Bam*HI digestion (see Figs. 1 and 2).

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Abbreviations: INC, integration-competent nucleoprotein complex; MoMLV, Moloney murine leukemia virus; LTR, long terminal repeat.

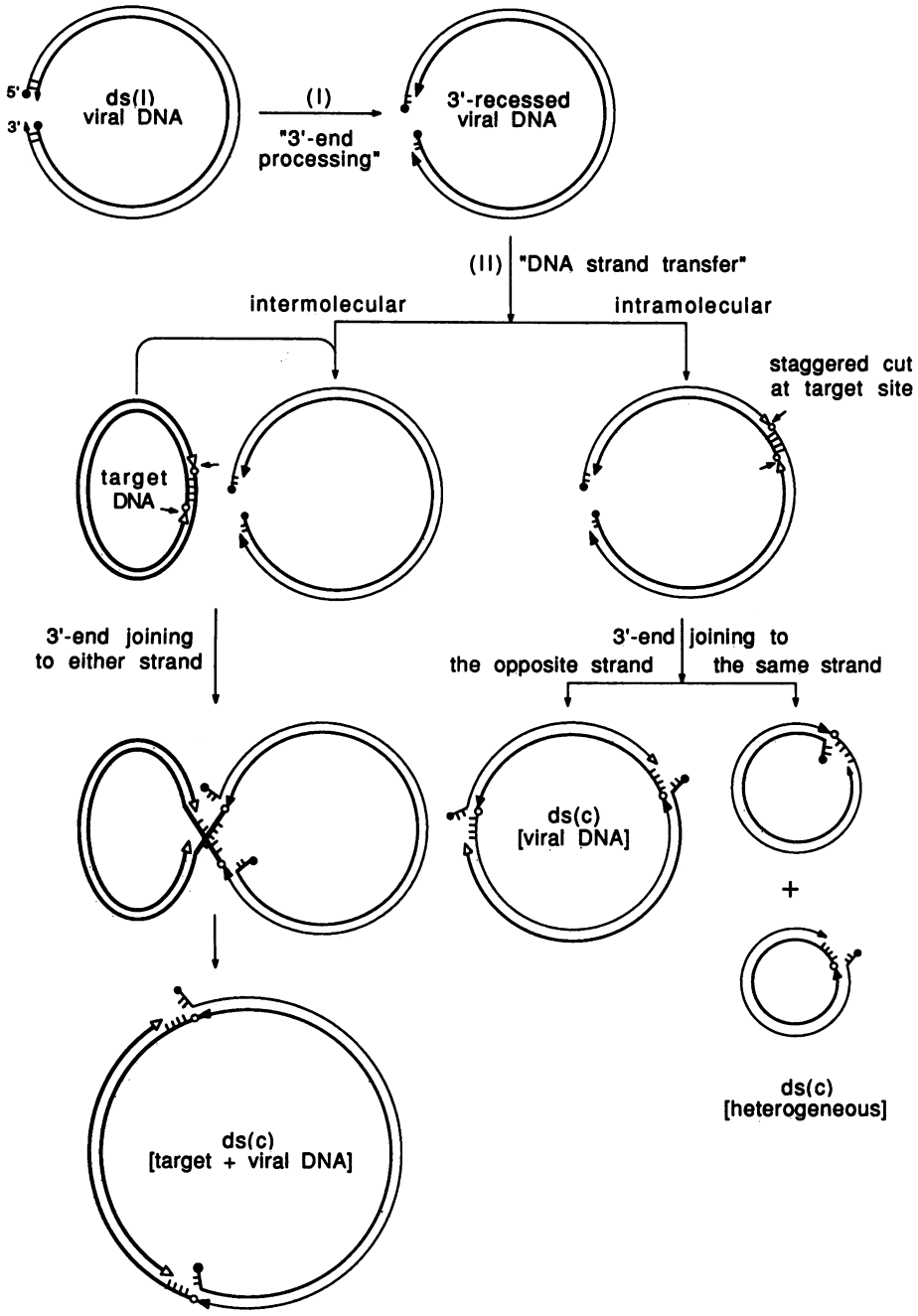

FIG. 1. Intermolecular and intramolecular retroviral DNA integration. In step I, two nucleotides are cleaved from each 3′ end of the double-stranded linear [ds(l)] viral DNA. Next (step II), a pair of intermolecular DNA strand transfer reactions join the resulting recessed 3′-OH ends (▲) of the viral DNA to the 5′ ends (○) of the target DNA at the site of integration. The 5′-phosphate ends (●) of the viral DNA and the 3′-OH ends (△) of the target DNA at the integration site remain unjoined. The resulting integration intermediate is a double-stranded circular [ds(c)] DNA with single-strand gaps at the junctions between the viral and target DNA sequences. Alternatively, the viral DNA can also undergo an intramolecular reaction in which the target sites for DNA strand transfer are on the viral DNA itself. When the 3′-OH ends (▲) of the viral DNA are joined to the 5′ ends (○) of the opposite strand at the target site, the integration intermediate will be a double-stranded circular DNA of the same length as the viral DNA, with an inversion of a DNA segment between one viral DNA end and the site of intramolecular integration. Alternatively, the 3′-OH ends (▲) of the viral DNA may join to the 5′ ends (○) of the same strand at the target site, giving rise to a pair of double-stranded circular DNA molecules; these products are heterogeneous because their size depends on the site of integration.

Disruption of the Autointegration Barrier by High Salt Treatment. To disrupt the autointegration barrier from the INC, fraction II was incubated on ice for 1 hr, after addition of KCl to 750 mM, unless indicated otherwise.

Fractionation of the Auto-INC by Velocity Sedimentation in Sucrose Gradients. To separate free components from the auto-INC, 0.2 ml of high-salt-treated fraction II containing the auto-INC was loaded onto a 2.0-ml sucrose gradient containing 20 mM Hepes·NaOH (pH 7.5), 5 mM $MgCl_2$, 6 mM EDTA, 150 mM KCl, and 15–30% sucrose, followed by centrifugation for 80 min at 210,000 × *g*. Gradients were fractionated from the top into 11 0.2-ml fractions. Each fraction from the gradient was subjected to the standard integration assay and the protein concentration was measured by the method of Bradford.

Reconstitution of the Autointegration Barrier. Standard reconstitution reaction mixtures (100 μl) containing 20 mM Hepes·NaOH (pH 7.5), 5 mM $MgCl_2$, 6 mM EDTA, 450 mM KCl, 0.02% bovine serum albumin, 3% sucrose, 40% Nycodenz (a nonionic density gradient medium, Accurate Chemical), and 15 amol (0.15 pM) of auto-INC were incubated on ice for 1 hr unless otherwise indicated and then subjected to the standard integration assay.

RESULTS

High Ionic Strength Promotes the Autointegration Reactions Catalyzed by the INC. As shown in Fig. 3*A*, the ratio of autointegration to intermolecular reaction products can be modulated by changing the ionic strength of the reaction mixture. At low ionic strength, integration was exclusively intermolecular, whereas autointegration was highly preferred at high ionic strength.

Does the preference for autointegration at high ionic strength result from loss of a barrier that protects the viral DNA from autointegration or inactivation of a positive factor that promotes the intermolecular reaction? Preliminary experiments established that autointegration is promoted by increasing the ionic

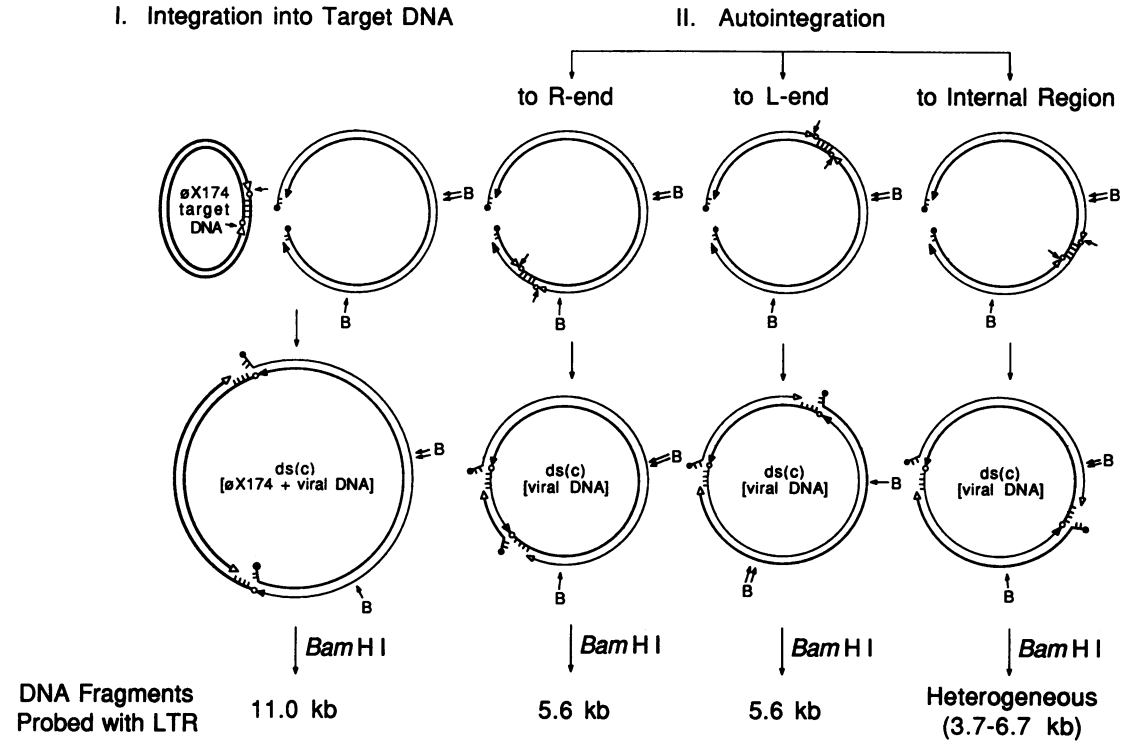

FIG. 2. Scheme for simultaneously analyzing intermolecular integration and autointegration reaction products. After incubating the INC with ϕX174 replicative form I DNA as a target, the reaction products are subjected to *Bam*HI digestion. All the intermolecular pairwise integration reaction products will yield 11.0-kb DNA fragments that hybridize with the LTR probe. Autointegration products that result from pairwise 3'-end joining to the opposite DNA strand at target sites (refer to Fig. 1) between the viral DNA ends and the *Bam*HI recognition sequences are visualized with the same probe as a 5.6-kb DNA fragment. The other autointegration products give rise to a smear of DNA fragments that are heterogeneous in size. Unreacted viral DNA will yield 3.7-kb (from L-end) and 1.9-kb (from R-end) LTR-containing DNA fragments. The 3.0-kb internal *Bam*HI fragment is not detected by the LTR probe.

strength of the reaction mixture even in the absence of ϕX174 replicative form I target DNA (data not shown). This supports the idea that high salt treatment disrupts a barrier that protects the viral DNA from autointegration.

Authenticity of the Autointegration Products. We confirmed that the 5.6-kb band was derived from autointegration reaction products by analyzing the products after further cleavage with *Xmn* I or *Bss*HII (Fig. 3*B*).

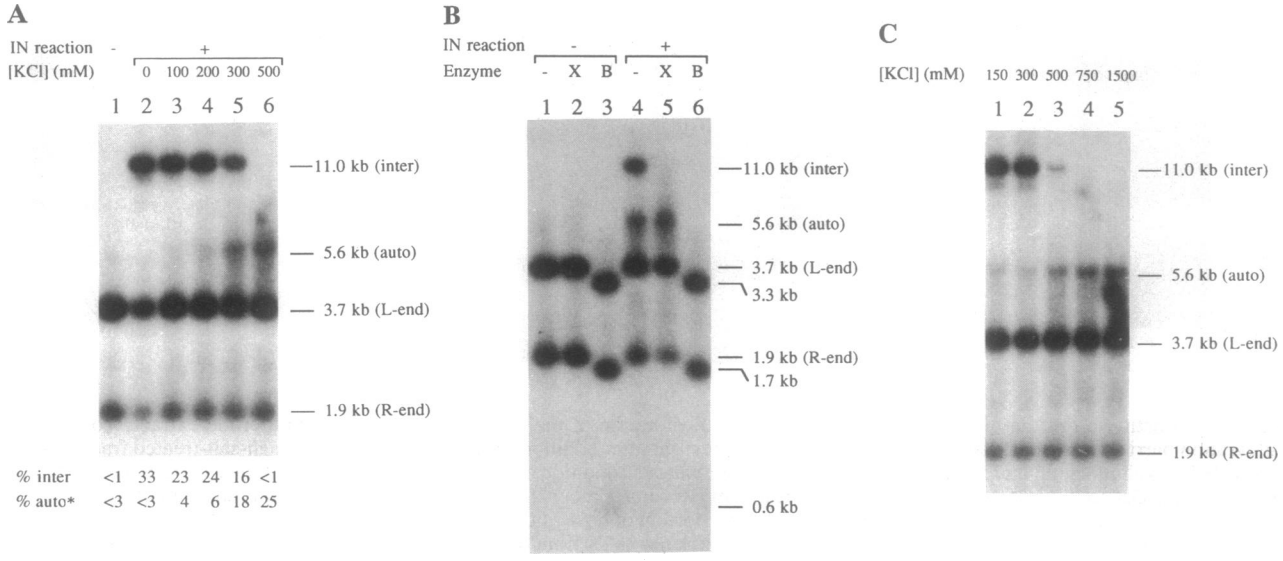

FIG. 3. Disruption of the INC autointegration barrier. (*A*) High ionic strength promotes the autointegration reaction catalyzed by the INC. The concentration of KCl was varied in the standard integration (IN) reaction mixture containing 10 mM $MgCl_2$ instead of $MnCl_2$ (lanes 2–6). The products were analyzed as described in Fig. 2. Lane 1 shows unreacted viral DNA. Values labeled % inter denote percentage of intermolecular integration products. Values labeled % auto* denote corrected percentage of autointegration products calculated as described in *Materials and Methods*. (*B*) Authenticity of the autointegration reaction products. Deproteinized viral DNA (lanes 1–3) or integration reaction products formed in the presence of 300 mM KCl and 10 mM $MgCl_2$ (lanes 4–6) were cleaved with *Bam*HI and then cleaved with a second restriction enzyme, *Xmn* I (lanes X) or *Bss*HII (lanes B). (*C*) Loss of the autointegration barrier after high salt treatment. Fraction II was preincubated on ice for 30 min after the addition of various concentrations of KCl, followed by the standard integration assay containing 200 mM KCl and 10 mM $MnCl_2$.

We first considered the possibility that the 5.6-kb DNA fragment might be generated from a circular form of viral DNA with two LTRs formed by end-to-end ligation of a linear viral DNA molecule. This was excluded by digestion with *Bss*HII, which cleaves once in the LTR region and would release a 0.6-kb DNA fragment specifically from the two-LTR circle. As shown in Fig. 3*B*, when the *Bam*HI-digested integration products were further cleaved with *Bss*HII, the 5.6-kb band disappeared, but the 0.6-kb band was not generated (compare lanes 4 and 6), as expected for autointegration products. A trace of the two-LTR circles was present in the INC preparation as indicated by the faint 0.6-kb band in lane 3. However, the intensity of this band did not increase after the integration reaction (compare the intensity of the 5.6-kb bands in lanes 1 and 4 with the intensity of the 0.6-kb digestion products in lanes 3 and 6). It should be noted that the 0.6-kb bands in both lanes 3 and 6 were faintly visible on the original autoradiogram.

An alternative possibility that putative one-ended intermolecular reaction products (ϕX174 double-stranded circular DNA with a branch) might comigrate with 5.6-kb fragments was ruled out by digestion with *Xmn* I, which cleaves the ϕX174 double-stranded circular DNA into three fragments. After *Xmn* I cleavage, the 11.0-kb intermolecular reaction products disappeared, as expected, since the sites of integration into the ϕX174 DNA are essentially random. However, the intensity of the 5.6-kb band remained the same (Fig. 3*B*, lane 5). One-ended intermolecular reaction products would be heterogeneous in size after cleavage by *Xmn* I and not be detected.

Disruption of the Autointegration Barrier by High Salt Treatment. To further investigate the effect of high salt, reactions were staged by preincubating the INC with various concentrations of KCl (Fig. 3*C*). Integration reactions were then initiated at 37°C by addition of $MnCl_2$ to 10 mM and target DNA; the KCl concentration was simultaneously adjusted to 200 mM. Autointegration events predominated when the INC was preincubated with high salt (KCl at 500 mM or more), even when reactions were subsequently carried out at low ionic strength. This suggests that the preincubation at high ionic strength causes a change in the organization of the INC that disrupts the barrier to autointegration. The resulting auto-INC may have lost protein components, undergone a conformational change, or both. We note that, consistent with a major structural change, high salt treatment results in a decrease in the sedimentation coefficient of the INC (data not shown).

Restoration of the Autointegration Barrier in a Crude Extract. Can the barrier to autointegration that is abolished by high salt treatment be regenerated? To test this possibility, the high-salt (750 mM KCl)-treated INC preparation was further incubated on ice after lowering the KCl concentration to 450 mM or 150 mM, with or without addition of Nycodenz to 50% (wt/vol) (Fig. 4*A*). The autointegration barrier was efficiently restored in the presence of both 450 mM KCl and Nycodenz (Fig. 4*A*, lanes 3 and 5). In the presence of 150 mM KCl, the autointegration barrier was partially restored, and the extent of restoration was not affected by Nycodenz (compare lanes 7 and 8 with lane 5). In the presence of 450 mM KCl, however, reconstitution was almost negligible (lane 6) but greatly enhanced by addition of Nycodenz (compare lanes 3 and 4 and also lanes 5 and 6) or by raising the concentration of the INC (compare lanes 4 and 6). The data suggest that a trans-acting factor or factors contribute to protecting the viral DNA against autointegration.

Separation of a Trans-Acting Factor from the Auto-INC. To obtain direct evidence that factors responsible for preventing autointegration are stripped from the INC by high salt

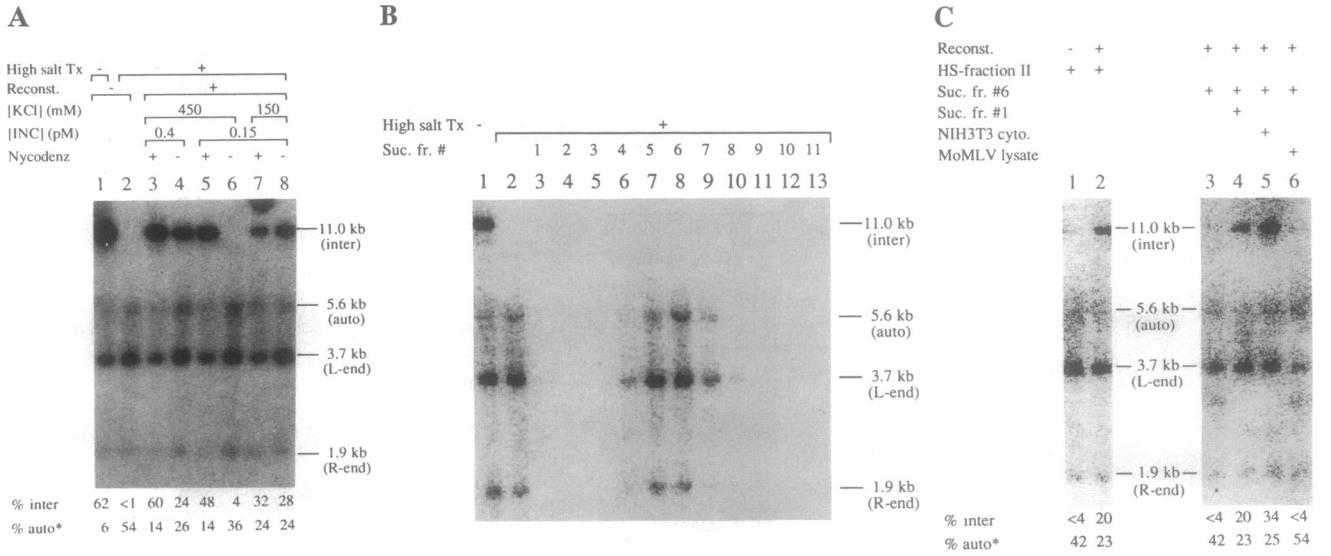

FIG. 4. Reconstitution of the barrier to autointegration. (*A*) Restoration of the autointegration barrier in a crude extract. To disrupt the autointegration barrier, fraction II (lane 1) was incubated on ice for 1 hr after addition of KCl to 750 mM. This high-salt-treated fraction II (lane 2) was divided into aliquots, further incubated on ice for 4 hr under various conditions as indicated (lanes 3–8), and then subjected to the standard integration assay. Data are presented as in Fig. 3. Tx, treatment; Reconst., reconstitution reaction. (*B*) Separation of the auto-INC from free proteins by velocity sedimentation in sucrose gradients. A 0.2-ml portion of high-salt-treated fraction II was loaded onto a 2.0-ml linear sucrose gradient (15–30% sucrose). Each sucrose-gradient fraction (Suc. fr.; lanes 3–13) from the gradient was subjected to the standard integration assay with 200 mM KCl. Lane 1 contains fraction II and lane 2 contains high-salt-treated fraction II. The auto-INC peak was fraction 6, whereas free proteins remained in the top fractions—50% in fraction 1 and 25% in fraction 2. (*C*) Reconstitution of the autointegration barrier onto the purified auto-INC. Lane 2 shows the reconstitution reaction of the unfractionated auto-INC [high-salt-treated (HS) fraction II] from 5×10^5 cells (4 μg of protein) in a standard reconstitution reaction mixture. Lanes 3–6 show reconstitution reactions of purified auto-INC (sucrose fraction 6 of Fig. 4*B*) with no additional proteins (lane 3), with sucrose fraction 1 of Fig. 4*B* (4 μg of protein; lane 4), with NIH 3T3 fibroblast cytoplasmic extract (cyto.; 20 μg of protein from 10^5 cells; lane 5), or with Nonidet P-40 (0.5%) lysate of MoMLV (20 μg of protein from 10^9 virus particles; lane 6). After incubation on ice for 1 hr, the reconstitution reaction products (lanes 2–6) and the unfractionated auto-INC without reconstitution (lane 1) were analyzed for their integration activities by the standard integration assay. Values labeled % auto* denote the corrected percentage autointegration.

treatment, we next attempted to physically separate such factors from the auto-INC by velocity sedimentation. The INC was treated with high salt and separated from free proteins by sedimentation in sucrose gradients (Fig. 4*B*). The sedimented auto-INC was unable to restore the barrier to autointegration when incubated alone in the presence of 40% Nycodenz (Fig. 4*C*, lane 3). However, the barrier to autointegration was restored when the sedimented complex was first mixed with a sucrose gradient fraction that contains free proteins (Fig. 4*C*, lane 4). The ability to separate the factor from the auto-INC provides an assay that can be used for its purification and identification.

The Trans-Acting Factor Is of Cellular Origin. Does the trans-acting factor come with the infecting virion or is it recruited from the host cell? Preliminary experiments have established that the factor is of cellular (not viral) origin. Host cell (NIH 3T3) cytoplasmic extract or a Nonidet P-40 lysate of MoMLV particles was assayed in the barrier-reconstitution assay (Fig. 4*C*). The host cell extract was shown (*i*) to contain a factor that reestablished the autointegration barrier (lane 5) and (*ii*) to be titratable in the barrier-reconstitution assay. The increase in intermolecular integration was equivalent to the decrease in autointegration (data not shown). In contrast, the Nonidet P-40 lysate of virus particles did not restore the autointegration barrier when added to the sedimented auto-INC complexes (lane 6). In addition, when partially purified virus-encoded proteins, including matrix, nucleocapsid, capsid, p12, and integrase, were individually tested in this assay, none of them supported reconstitution of the autointegration barrier (data not shown).

DISCUSSION

The INC isolated from retrovirus-infected cells provides a tool to analyze aspects of the retroviral integration reaction that are not reproduced with purified integrase protein and simple DNA substrates. The high efficiency and fidelity of *in vitro* integration reactions with the INC allow the physical properties of the complexes to be readily correlated with function. By biochemically analyzing the INC of MoMLV, we have demonstrated the existence of a barrier that protects the retroviral DNA against autointegration. The barrier can be abolished by incubating the complexes at high ionic strength, which causes reversible dissociation of a factor(s) that can be provided as a host cell extract. How does the factor block autointegration? Simple possibilities include physically blocking integration by coating the viral DNA and/or promoting condensation of the viral DNA.

We cannot strictly conclude that the factor supplied by extracts of uninfected cells is identical to the factor removed from the INC by high salt treatment. However, the similar behavior in the reconstitution assay of the authentic factor separated from the INC by sedimentation in sucrose and the factor supplied as uninfected cell extract supports this hypothesis. The assay system described here provides a means to purify the factor from extracts of uninfected host cells and determine its identity.

Is the host factor packaged in the virion or acquired after cell entry? Our results suggest that the factor is not present in the virion and is, therefore, likely to be incorporated into the INC after infection. It is tempting to speculate that viral proteins associated with the DNA play a role in recruiting the host factor. A nonspecific DNA binding protein alone would not be expected to discriminate viral DNA from host DNA. Although our results suggest that the factor is of cellular origin, we note that NIH 3T3 cells contain many endogenous sequences related to MoMLV. The failure of virion proteins to support reconstitution argues against a viral gene origin but does not strictly exclude the possibility that a factor present in virions might be modified after cell entry.

We speculate that other retroviruses also possess a defense against autointegration. Like MoMLV, human immunodeficiency virus INC also preferentially integrates into an intermolecular target (11, 12). Rather different results have been reported for the INC of avian leukosis virus, which predominantly undergoes autointegration *in vitro* (9). Unlike MoMLV INC, the avian leukosis virus complexes contain incomplete reverse transcripts that must be completed by *in vitro* synthesis prior to *in vitro* integration. Lee and Coffin (10) suggested that reverse transcription may not be completed *in vivo* until the complexes are physically juxtaposed with target DNA, thus avoiding the predominantly autointegration pathway observed *in vitro*. It is also possible that the avian leukosis virus complexes are more labile than those of MoMLV and lose a factor(s) during isolation that normally protects the viral DNA from autointegration in the cell.

Although a great deal has been learned about the enzymology of integrase protein itself, much remains to be understood about the complete integration process. Identification of the host factor described here and elucidation of its role and mode of action should further our understanding of the preintegration steps in the retroviral replication cycle and may point to therapeutic agents for blocking viral replication.

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