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Integration of human immunodeficiency virus type 1 DNA *in vitro*

(retrovirus/AIDS)

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ABSTRACT **A highly efficient cell-free system for the integration of human immunodeficiency virus type 1 DNA is described. Linear viral DNA synthesis occurs in the cytoplasm of newly infected cells, reaching peak levels 4 hr after infection. The linear viral DNA molecules present in cytoplasmic extracts are capable of integrating into heterologous DNA targets *in vitro*. The viral DNA resides in a high molecular weight nucleoprotein structure that can be separated from the bulk of cellular protein and nucleic acid without a detectable decrease in the ability to integrate *in vitro*.**

Two events in the replication cycle distinguish retroviruses from other classes of virus, synthesis of a double-stranded DNA molecule from a single-stranded RNA genome and integration of this DNA intermediate into the genome of the host cell. Shortly after entry into the cytoplasm of a susceptible cell, the single-stranded RNA genome is converted by the viral enzyme reverse transcriptase into a linear double-stranded DNA molecule that is flanked by directly repeated sequences termed long terminal repeats (LTRs) (1). The viral DNA intermediate then migrates to the nucleus, where it is covalently integrated into a host chromosome. After integration, the viral DNA is transcribed into RNA to be used either as mRNA for the synthesis of viral proteins or as genome RNA for progeny virions.

The structural and enzymatic functions involved in the integration of retroviral DNA are not yet understood. Only the viral integrase protein has been shown to play a direct role in the integration of viral DNA. The role of other viral proteins in this reaction and the nature of the other functions necessary for the integration of the provirus, such as cleavage of the cellular DNA target and ligation of viral DNA to cellular DNA, remain to be determined. The identification of the proteins required for the integration reaction requires the development of cell-free systems that support the integration of viral DNA *in vitro*. Such a system is described here for the integration of DNA of the human immunodeficiency virus type 1 (HIV-1).

MATERIALS AND METHODS

Cells and Viruses. The MOLT IIIB cell line was used as a source of virus for cell-free infections. This chronically infected human T-cell line constitutively produces a low level of the HTLV-IIIB strain of HIV-1. Virus production from these cells can be stimulated approximately 10-fold by incubation with phorbol esters. MOLT IIIB cells were cultured for 24 hr in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum and phorbol 12-myristate 13-acetate (10 ng/ml) at a cell density of 1–2 × 10^6 per ml. Supernatant from stimulated cells was collected and filtered through a 0.2-μm (pore size) cellulose acetate filter. Virus was pelleted by centrifugation of filtered supernatants for 1 hr at 25,000 × *g*. Pelleted virus was gently resuspended in a minimal volume of RPMI 1640 medium plus 10% fetal calf serum and used to infect cells of the SupT1 human T-cell line.

Preparation of Cell Extracts. SupT1 cells were infected with concentrated virus (14–18 μg of p24 viral core protein per ml) at 4 × 10^6 cells per ml. Cell extracts were prepared by a modification of the procedure of Brown *et al.* (2). At specified times after infection, cells were washed twice with buffer K (20 mM Hepes, pH 7.4/150 mM KCl/5 mM $MgCl_2$/1 mM dithiothreitol/25 mM aprotinin) and then lysed at a concentration of 33 × 10^6 cells per ml in the same buffer containing 0.025% digitonin or 0.025% Triton X-100. Cells were extracted for 10 min at room temperature, and the lysate was centrifuged at 1000 × *g* for 3 min. The supernatant, after clarification by centrifugation at 8000 × *g* for 3 min, is referred to as "cytoplasmic extract." This cytoplasmic extract was the source of viral DNA for analysis of the time course of DNA synthesis and for the *in vitro* integration reaction. The pellet from the initial low-speed centrifugation contained cell debris and intact nuclei and is called the "nuclear pellet."

***In Vitro* Integration Reaction.** *Pst* I-linearized (replicative form III) ϕX174 DNA, relaxed circular (replicative form II) ϕX174 DNA, and single-stranded M13 DNA were used as targets for the *in vitro* integration of viral DNA. Target DNA was added to cytoplasmic extracts of infected SupT1 cells to a concentration of 10 μg/ml, and reaction mixtures were incubated at 37°C. Reactions were stopped by the addition of SDS and proteinase K (see below).

DNA Preparation. Viral DNA present in cytoplasmic extracts was deproteinated by incubation with proteinase K (1 mg/ml)/10 mM EDTA/0.5% SDS for 1 hr at 55°C. Samples were extracted successively with phenol/chloroform [1:1 (vol/vol)] and chloroform. DNA was ethanol-precipitated, washed with 70% ethanol, resuspended in 10 mM Tris·HCl (pH 7.4), and treated with RNase A (20 μg/ml) for 1 hr at 37°C before analysis by agarose gel electrophoresis and Southern blotting.

For analysis of unintegrated viral DNA in the nucleus of infected cells, low molecular weight DNA was isolated from nuclear pellets of cell extracts by the method of Hirt (3). Samples were treated with RNase A (20 μg/ml) for 30 min at 37°C, extracted with phenol/chloroform and then with chloroform, and precipitated with ethanol.

Southern Blot Analysis. DNA samples were layered on 0.8% agarose gels and electrophoresed at 1 V/cm for 15 hr in Tris/acetate/EDTA buffer (4). After electrophoresis, DNA was blotted by capillary action onto nitrocellulose (0.45 μm, Schleicher & Schuell). Filters were hybridized in formamide/dextran sulfate buffer by using standard methods (4). A 720-base-pair fragment of the HIV-1 LTR spanning the unique *Xho* I site and the *Hin*dIII site present in the R region of the HXBc2 molecular clone of HIV-1 was used as a probe for hybridization. The probe was labeled with [^{32}P]dCTP

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Abbreviations: LTR, long terminal repeat; HIV-1, human immunodeficiency virus type 1; MLV, murine leukemia virus.

(>6000 Ci/mmol; 1 Ci = 37 GBq; NEN/DuPont) by the random hexamer labeling procedure using a commercially available kit (United States Biochemicals).

RESULTS

Time Course of Viral DNA Synthesis. A synchronous infection system was used to study the synthesis of HIV-1 viral DNA *in vivo*. To achieve a synchronous infection, concentrated virus was placed onto rapidly dividing SupT1 cells, a human T-cell line that is highly susceptible to HIV-1 infection by virtue of a high surface density of the virus receptor CD4 molecule. Viral DNA formation was followed by Southern blot analysis of cytoplasmic extracts prepared from cells at various times after infection. Linear viral DNA was first detected approximately 2 hr after infection and reached peak levels between 4 and 6 hr (Fig. 1*A*). Between 8 and 15 hr after infection, the levels of linear DNA in the cytoplasm decreased, concurrent with the appearance of increasing levels of viral DNA in the nucleus. Viral DNA forms appeared in the nucleus as early as 4 hr after infection, with approximately equal amounts of linear and covalently closed circular (one LTR) forms present (Fig. 1*B*). By 10 hr after infection, all forms of viral DNA (linear, covalently closed circles with one LTR or two LTRs, and open circles with one LTR or two LTRs) were present in the nucleus (Fig. 1*B*). At no time were circular forms of viral DNA detectable in the cytoplasm, indicating that circularization of viral DNA occurred exclusively in the nucleus.

***In Vitro* Integration of Viral DNA.** The ability of the linear viral DNA present in the cytoplasm of cells newly infected with HIV-1 to integrate into heterologous DNA targets *in vitro* was determined. Cytoplasmic extracts were prepared from SupT1 cells 4–5 hr after infection with concentrated virus, at a time when viral DNA was near peak levels. Integration into a heterologous DNA target was detected by the shift of linear viral DNA on Southern blots from the characteristic 9.7-kilobase (kb) position to a position corresponding to viral DNA plus target DNA. Fig. 2 (lane e) shows the product of integration of viral DNA into the linear form (replicative form III) of bacteriophage ϕX174 DNA. The position of the upper band in this lane corresponds to a molecule of a size consistent with the integration of linear HIV-1 DNA into a 5.4-kb target. Fig. 2 (lane g) shows the linear product of integration into relaxed circular (replicative form II) ϕX174 DNA after digestion with the restriction enzyme *Aat* II, which cuts once in ϕX174 DNA sequences and does not cut HIV-1 DNA. Densitometric scanning of the blots indicates that greater than 98% of the viral DNA molecules have integrated into the linear or relaxed circular targets after a 1-hr incubation. The time-course results demonstrate that the amount of integration products increased progressively during the incubation at 37°C (Fig. 2, lanes a–e). The reaction was complete after 45 min of incubation. Hybridization of reaction products with a ϕX174-specific probe indicated that target DNA sequences were present in the 15-kb integration product (data not shown). The possibility that the integration product resulted from the end-to-end ligation of viral DNA and target DNA is not consistent with the results of integration into a relaxed circular DNA target, which contains no free ends. Furthermore, pretreatment of linear target DNA with alkaline phosphatase had no effect on the *in vitro* integration reaction (data not shown). Incubation of purified linear viral DNA with a linear DNA target in cytoplasmic extracts prepared from uninfected SupT1 cells failed to result in the formation of integration product (Fig. 2, lane f).

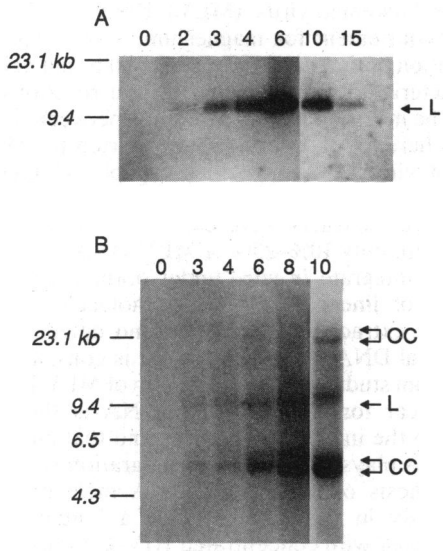

FIG. 1. Time course of viral DNA synthesis in newly infected cells. Southern blot hybridization analysis of unintegrated viral DNA present in cytoplasmic (*A*) and nuclear (*B*) extracts of infected cells. Numbers at the top of the lanes indicate hours after infection. Arrows point to viral DNA species. OC, L, CC, open circular, linear, and closed circular DNAs, respectively.

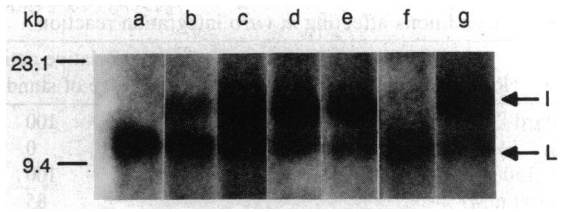

FIG. 2. *In vitro* integration of linear HIV-1 DNA. Cytoplasmic extracts prepared from SupT1 cells 4 hr after infection with HIV-1 were incubated with exogenously added target DNA under integration reaction conditions. Products of the reactions were analyzed by Southern blot hybridization. Time course of integration into linear ϕX174 DNA target. Target DNA was added to the extract and the reaction was stopped after 0, 5, 15, 30, or 45 min of incubation at 37°C (lanes a–e, respectively). Purified linear viral DNA was incubated for 1 hr at 37°C with cytoplasmic extract prepared from uninfected SupT1 cells (lane f). Integration into open circular ϕX174 DNA target; reaction product was linearized by digestion with the restriction endonuclease *Aat* II, which cleaves once in ϕX174 DNA sequences and does not recognize sequences in HIV-1 (lane g). Arrows indicate the positions of viral DNA products. L, linear viral DNA: I, integration product.

Table 1 outlines conditions that influenced the efficiency of the *in vitro* integration reaction. Integration *in vitro* occurred optimally at a monovalent cation concentration of 150 mM. Concentrations of KCl of 250 mM or greater completely abolished detectable integration. An absolute requirement for magnesium for integration *in vitro* was demonstrated by the absence of detectable integration in buffer devoid of added $MgCl_2$ and containing 1 mM EDTA (see Fig. 4*B*, lane f). Integration occurred with equal efficiency at pH values ranging from 7.0 to 8.0, suggesting a broad pH optimum for the reaction. The integration activity was heat labile, as pretreatment of cytoplasmic extracts to 60°C for 5 min prior to incubation at 37°C completely abolished integration. Pretreatment of extracts with proteinase K prior to incubation with target DNA also abolished integration *in vitro*, further evidence that protein components of the extract are necessary for the integration of viral DNA. The single-stranded M13mp18 plasmid DNA did not serve as a target for *in vitro* integration, suggesting a requirement for a double-stranded target in the integration reaction (data not shown).

Characterization of the *in Vitro* Integration Complex. Cytoplasmic extracts from HIV-1-infected SupT1 cells were

Table 1. Conditions affecting *in vitro* integration reaction

Reaction condition(s)	Relative activity, % of standard
Standard	100
− Mg^{2+}/+ EDTA	0
NaCl (150 mM)	100
KCl (100 mM)	85
KCl (250 mM)	0
pH 7.0	100
pH 8.0	100
60°C/5-min preincubation	0
Proteinase K pretreatment/37°C/30 min	0
RNase A pretreatment/37°C/30 min	100
Open circular DNA target	100
Single-stranded DNA target	0
Uninfected cell extract	
+ purified linear viral DNA	
+ linear DNA target	0

Target DNA was present at 10 μg/ml in all samples. Extent of integration was determined by densitometric analysis of autoradiograms of Southern blots of integration reaction products.

analyzed by sucrose gradient sedimentation and gel-filtration chromatography to determine the physical properties of the *in vitro* integration activity. Fig. 3*A* shows the Southern blot detection of viral DNA in fractions collected from a 15%–30% (wt/vol) sucrose gradient. Viral DNA concentrations peaked near the middle of the gradient. Fractions containing viral DNA also contained the bulk of cellular rRNA, as detected by absorbance at 260 nM and agarose gel electrophoresis, indicating that the integration complex has a sedimentation profile similar to that of a ribosome. Viral DNA present in the gradient fractions was fully competent for integration *in vitro* into linear DNA targets (Fig. 3*B*). Linear viral DNA, prepared from infected cells and deproteinated by treatment with proteinase K and phenol extraction prior to sedimentation, did not enter the sucrose gradient. Purified viral DNA mixed with cytoplasmic extracts prepared from uninfected SupT1 cells also failed to enter the sucrose gradient (Fig. 3*C*). Prior treatment of extracts with RNase A had no effect on the migration of viral DNA on sucrose gradients (data not shown).

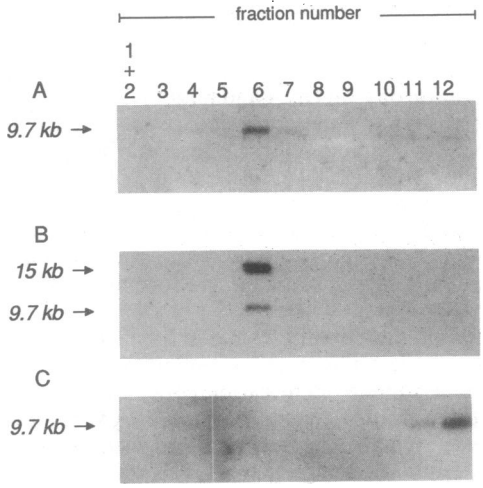

FIG. 3. Sucrose gradient sedimentation of viral DNA in cytoplasmic extracts. (*A*) Cytoplasmic extracts (1 ml) prepared from SupT1 cells 4 hr after infection were layered onto 12-ml gradients of 15%–30% sucrose in buffer K and centrifuged at 35,000 rpm for 3 hr at 4°C in a Beckman SW 41 rotor. Fractions (1 ml) were collected from the bottom of the gradient, deproteinated, and assayed for viral DNA by electrophoresis and Southern blot hybridization. (*B*) *In vitro* integration of viral DNA in gradient fractions. Conditions were the same as described in *A*, except that linearized ϕX174 DNA was added to gradient fractions to 10 μg/ml and incubated at 37°C for 45 min prior to deproteination. (*C*) Sedimentation of deproteinated viral DNA. Purified linear HIV-1 DNA was mixed with 1 ml of cytoplasmic extract from uninfected SupT1 cells and subjected to sedimentation as in *A*. Numbers above the lanes indicate fraction numbers. Fraction 1 is the bottom of the gradient; fraction 12 is the top. Arrows indicate the positions of linear viral DNA (9.7 kb) and integration products (15 kb).

Viral DNA present in the cytoplasmic extracts was separated from the bulk of cellular and viral proteins by Sephacryl S-1000 column chromatography. Southern blot analysis of the fractions collected from the column shows that the viral DNA eluted near the void volume, well separated from the peak of cellular protein eluted from the column (Fig. 4 *A* and *C*). Determination of protein concentration in each fraction indicated that the fractions with peak levels of viral DNA contained less than 1% of the total protein eluted from the column. The major viral core protein p24, detected by radioimmunoassay of column fractions, eluted from the column along with the bulk of cellular protein (Fig. 4*C*), and fractions that contained peak levels of viral DNA had no viral p24 detectable by this assay. Chromatography of the viral integration complex had no effect on the ability of the viral DNA to integrate *in vitro*. Fig. 4*B* shows that greater than 98% of the viral DNA molecules present in the peak fractions from the column were capable of integrating into a linear DNA target *in vitro*.

DISCUSSION

Newly synthesized viral DNA present in extracts of cells infected with HIV-1 was shown to be part of a nucleoprotein complex capable of mediating the integration of viral DNA into heterologous DNA targets *in vitro*. Greater than 98% of the viral DNA molecules present in the cytoplasm of cells infected 4 hr earlier were capable of integrating into a DNA target *in vitro*, providing the most efficient assay to date for retroviral integration. The nucleoprotein complex is necessary for integration as deproteinated viral DNA was incapable of integrating into a DNA target when added exogenously to cytoplasmic extracts from uninfected cells.

The conditions for the HIV-1 *in vitro* integration reaction reported here are similar to those reported for the integration of murine leukemia virus (MLV) DNA *in vitro* (2). An absolute requirement for magnesium, a monovalent cation concentration optimum of 150 mM, and a broad pH optimum are characteristic of *in vitro* integration reactions for both viruses. The most significant difference between the reaction described here for HIV-1 and that reported for MLV is the fraction of viral DNA molecules capable of integrating *in vitro*. Whereas nearly all of the HIV-1 DNA molecules present in cell extracts were capable of integrating into a target *in vitro*, only 10%–25% of MLV DNA molecules were reported to integrate *in vitro* under optimal conditions (5). Integration of linear HIV-1 DNA molecules occurred in cytoplasmic extracts that contained no detectable circular forms of viral DNA. This observation is consistent with the evidence from studies of the integration of MLV DNA *in vitro* that the linear form of retroviral DNA is the immediate precursor to the integrated provirus and that circularization of viral DNA plays no role in the integration reaction (5, 6).

The synthesis of linear viral DNA molecules occurred synchronously in the cytoplasm of a human T-cell line acutely infected with concentrated HIV-1. Linear viral DNA was first detected in the cytoplasm approximately 2 hr after infection, and peak levels occurred between 4 and 6 hr. Thereafter, viral DNA levels in the cytoplasm began to decrease, so that, by 15 hr after infection, unintegrated viral DNA was present at less than 10% of peak levels (Fig. 1*A*). At the same time that cytoplasmic unintegrated viral DNA

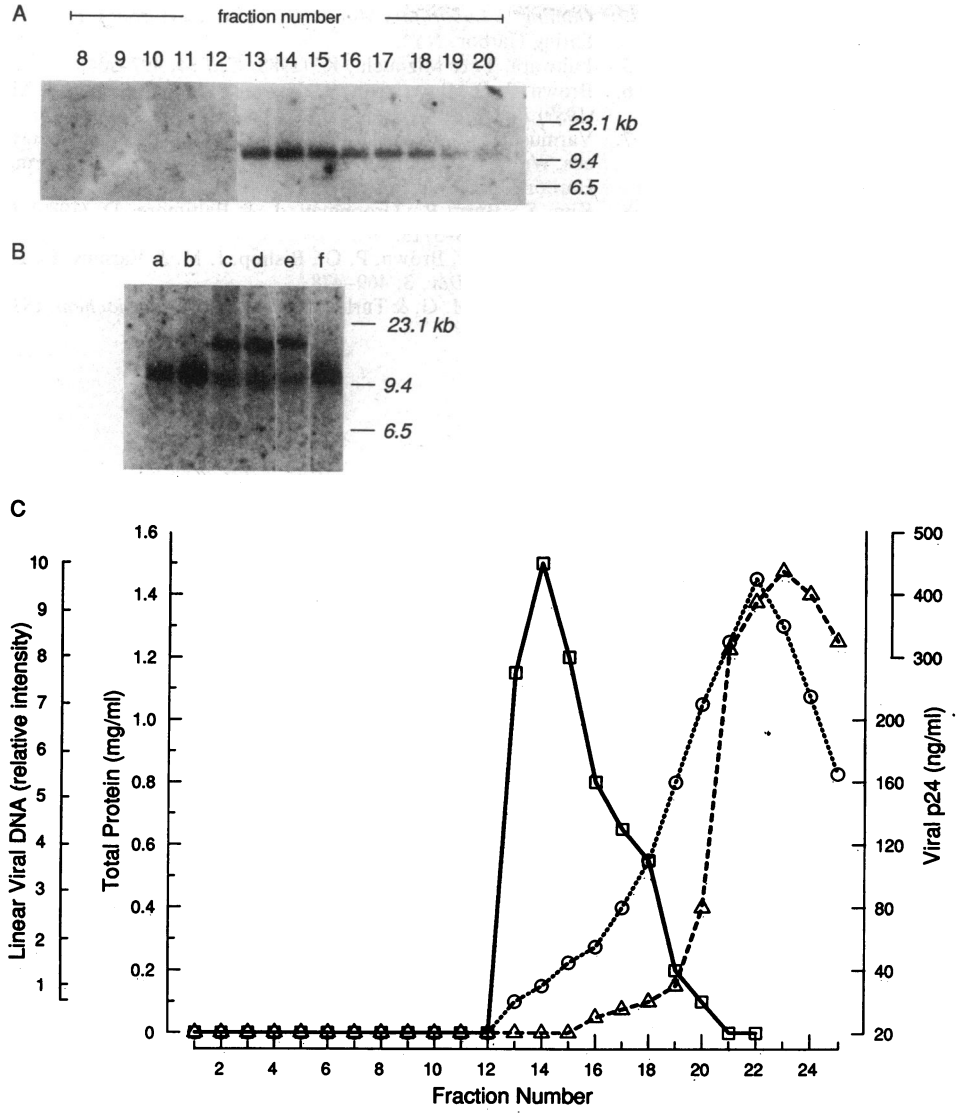

FIG. 4. Sephacryl S-1000 chromatography of viral DNA in cytoplasmic extracts. (*A*) Cytoplasmic extracts (1 ml) were chromatographed on a 7-ml Sephacryl S-1000 (Pharmacia) column equilibrated in buffer K. Fractions (0.3 ml) were collected, deproteinated, and assayed for viral DNA by gel electrophoresis and Southern blot hybridization. Numbers above the lanes indicate fractions from the column. (*B*) *In vitro* integration of viral DNA in column fractions. Lanes: a, viral DNA from extract prior to chromatography; b, same as lane a, except incubated at 37°C for 45 min; c, d, and e, column fractions 13, 14, and 15, respectively, after incubation with linear ϕX174 (10 μg/ml) at 37°C for 45 min; f, *in vitro* integration reaction of viral DNA in fraction 13 of chromatography as in *A*, except that $MgCl_2$ was omitted from the chromatography buffer, and 1 mM EDTA was added to the reaction mixture. (*C*) Profile of linear viral DNA (□), viral p24 (△), and total protein (○) eluted from the Sephacryl S-1000 column. Amount of viral DNA present per fraction is expressed as relative densitometric intensity of bands corresponding to integration products on autoradiogram shown in *A*. Viral p24 concentration was determined by the HIV-1 strain HTLV-III p24 radioimmunoassay (DuPont). Total protein concentration in each fraction was determined by the BCA protein reagent microassay (10).

levels were decreasing, viral DNA became detectable in the nuclear fractions of such extracts. Only the linear form of unintegrated viral DNA was detectable in the cytoplasm, whereas both linear and circular forms were present in the nucleus from the earliest times of detection. Such a course of viral DNA synthesis and nuclear migration is similar to that observed for other retroviruses (7) and is in agreement with results reported for HIV-1 (8).

Experiments with MLV have shown that linear viral DNA present in the cytoplasm of infected cells is complexed with proteins in a high molecular weight nucleoprotein structure (9). Likewise, unintegrated linear viral DNA in cells newly infected with HIV-1 migrated on sucrose gradients as if it were contained in a high molecular weight structure. If the extracts were deproteinated prior to sedimentation, the viral DNA did not enter the gradient, indicating that protein components contribute to the high sedimentation rate of the structure. Pretreatment of extracts with RNase A prior to sedimentation had no effect on the migration of viral DNA, suggesting that RNA is not an integral part of the integration complex. The nucleoprotein structure identified by sucrose gradient sedimentation may contain all the functions necessary for the integration of viral DNA into a DNA target, as viral DNA present in gradient fractions was fully capable of integrating *in vitro*. However, the possibility remains that cellular factors necessary for the integration reaction copurify with the viral integration complex.

Purification of viral integration complexes that retain full integration activity is the first step toward identifying the protein components of the complex. Gel-filtration chromatography of cell extracts allowed a substantial purification of the integration complex without a loss of *in vitro* integration activity. The fractions containing integration activity were depleted of greater than 99% of the total protein in the cellular extract. Furthermore, integration activity was clearly separable from the bulk of viral p24. The latter observation suggests that most of the p24 carried into the cell by the infecting virions is either not complexed with viral DNA or can be dissociated from the viral DNA without a loss of integration activity. This observation does not rule out the possibility that some of the viral capsid proteins, including p24, are associated with the preintegration complex.

Previous assays for retroviral integration have been limited by the low fraction of viral DNA molecules capable of integrating into the target molecule. Such a low efficiency of integration, combined with an inability to distinguish between complexes capable of integration from those unable to integrate, has complicated attempts to determine the composition of an integration competent nucleoprotein complex. The development of an *in vitro* reaction that supports the integration of essentially all of the viral DNA molecules in an extract should greatly facilitate the task of further identifying the protein factors necessary for the integration of retroviral DNA.

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