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Human Immunodeficiency Virus Type 1 2-LTR Circles Reside in a Nucleoprotein Complex Which Is Different from the Preintegration Complex

MICHAEL BUKRINSKY,[1]* NATALIA SHAROVA,[2] AND MARIO STEVENSON[2]

The Picower Institute for Medical Research, Manhasset, New York 11030,[1] and Department of Pathology, University of Nebraska Medical Center, Omaha, Nebraska 68198-5120[2]

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The preintegration complex of human immunodeficiency virus type 1 (HIV-1) is a large nucleoprotein complex containing viral nucleic acids in association with products of the viral *gag* and *pol* genes. One of these proteins, integrase, is absolutely required for the integration and formation of the provirus. Although HIV-1-specific 2-LTR circles from nuclei of HIV-1-infected cells were found to be associated within a high-molecular-weight nucleoprotein complex, antibodies to HIV-1 integrase failed to precipitate this form of viral DNA. This result indicates that circular forms of HIV-1 DNA are not associated with integrase. These viral DNA forms seem to exist in a context of a nucleoprotein complex that is different from a preintegration complex of HIV-1.

Acute infection with retroviruses results in a variety of DNA molecules derived from the infecting viral RNA genome. Reverse transcription of the human immunodeficiency virus type 1 (HIV-1) genome is initiated in the cytoplasm and proceeds concurrently with the nuclear import of the viral preintegration complex (5), leading to the formation of a linear, double-stranded DNA molecule bounded by directly repeated sequences termed long terminal repeats (LTRs) (18). Inside the nucleus two other forms of HIV-1 DNA can be detected: covalently closed circular forms with one LTR (products of recombination) or two tandem LTRs (resulting either from ligation of the LTR ends, producing a circle junction with GTAC between the LTR termini, or from autointegration which produces significantly rearranged molecules) (7, 9). Recent studies with a number of retroviral systems have indicated that the linear viral DNA is the precursor to the integrated provirus (2, 3, 8), while circular forms are a dead-end product (3, 10). Although these viral DNA forms are unable to integrate and support viral replication, they may play a significant role in HIV cytopathogenesis. Several reports have associated the accumulation of circular molecules with HIV-1 cytotoxicity (11, 13, 17a). The basis for this phenomenon may be the ability of extrachromosomal circular viral DNA to support low levels of protein expression (17, 19), although this concept is questioned in another report (15).

Factors which regulate the balance of integrated to extrachromosomal circular viral DNA are not well understood. Circularization via autointegration competes with integration (7), since both of these reactions utilize the same preintegration complex and rely on the virus-encoded integrase. In an HIV-infected cell, this balance is shifted toward integration because of high concentrations of target DNA in the nucleus (7). Circularization by recombination or ligation does not compete with integration (7).

Indeed, in vivo results indicate that circular molecules formed by ligation or recombination are more abundant than products of autointegration (9, 12). Reported data on the biological activity of extrachromosomal viral DNAs were obtained with integrase-deficient viruses (17, 19), and therefore DNA circles in those experiments (4) could have arisen only from ligation or recombination and not from self-integration. Since these simple circles also contain higher numbers of intact, nonrearranged HIV genomes (7, 9, 12) and therefore would be the most likely candidates for the role played by extrachromosomal viral DNA in pathogenesis, we asked in what form do these molecules exist in vivo.

A total of 2×10^8 to 3×10^8 MT-4 cells infected with HIV-1 mfA (16) were swelled in a hypotonic buffer and lysed by 30 strokes of a type B pestle in a Dounce homogenizer (4, 5). High-molecular-weight nucleoprotein complexes were then released from the nuclei by a high-salt extraction buffer, exactly as described previously (4, 5). After fractionation on a Nycodenz density gradient according to the previously described procedure (1, 4, 5), fractions were dialyzed against 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.9)–20% glycerol–100 mM KCl–1 mM phenylmethylsulfonyl fluoride and analyzed by polymerase chain reaction (PCR) for the presence of linear and circular forms of viral DNA and for in vitro integration activity (4, 5). Two hundred microliters of the total volume of 800 μl for each fraction was taken for the DNA analysis, while the rest was used for an in vitro integration assay. The density of the fractions was calculated by using the formula, density = $0.135 \times OD_{360} + 1.0$, where OD_{360} is the optical density at 360 nm; density was measured in grams per milliliter (1). The linear and circular forms of viral DNA as well as in vitro integration activity were found to peak around the gradient fraction with a density of 1.36 g/ml (Fig. 1). To exclude the possibility that this peak simply represents chromatin fragments, we performed PCR with primers specific for the α-tubulin gene (4). This experiment demonstrated that genomic DNA did not form any major peak with a density close to 1.36 g/ml (Fig. 1A), indicating that nuclear extracts were not significantly contaminated with chromatin fragments. The density of HIV-1 preintegration complexes obtained in this experiment is higher than 1.20 g/ml, which was reported recently for HIV-1 preintegration complexes (4), and 1.26 g/ml, which was reported for murine leukemia virus preintegration complexes (1). We attribute this

* Corresponding author.

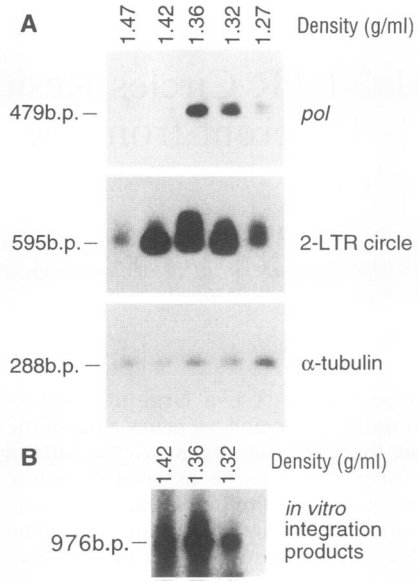

FIG. 1. Equilibrium density gradient fractionation of HIV-1 nucleoprotein complexes. Gradient fractions were analyzed for the presence of in vitro integration activity by a PCR-based assay (4, 5) (B) and for the presence of HIV-1 DNA by PCR with primers specific for the HIV-1 *pol* gene or 2-LTR circles (4, 5) (A). Cellular α-tubulin gene amplification was used as a control.

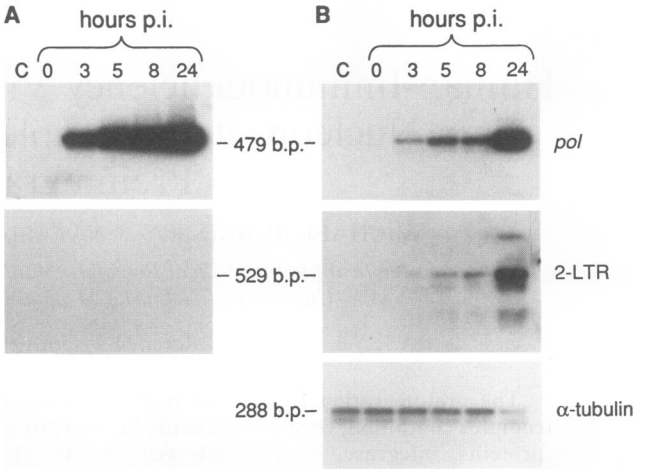

FIG. 2. Analysis of HIV-1 DNA in the nuclei of infected MT-4 cells. (A) Immunoprecipitation of HIV-1-specific nucleoprotein complexes with monoclonal antibodies to integrase. HIV-1 DNA extracted from immunoprecipitates was analyzed either by one round of PCR with primers specific for the *pol* gene or by two rounds with nested primers specific for 2-LTR circles (4, 5). C, uninfected MT-4 cells. (B) HIV-1 2-LTR circle accumulation in the nuclei of infected MT-4 cells. One-tenth of the nuclear extract used for panel A was analyzed without immunoprecipitation by one round of PCR with HIV-1 *pol*- or cellular α-tubulin-specific primers or by two rounds with nested primers specific for 2-LTR circles (4, 5).

difference to the inconsistency of Nycodenz gradients, probably due to different states of hydration of the nucleoprotein complexes in the nonionic medium (1). The result of this experiment indicated that HIV-1 2-LTR circular DNA was associated with a high-molecular-weight nucleoprotein complex. Similar experiments localized 1-LTR circular DNA to the same fractions of the gradient (data not shown).

These complexes either represented bona fide preintegration complexes or copurified with those complexes on a density gradient. To resolve this question we immunoprecipitated virus-specific nucleoprotein complexes with monoclonal antibodies to HIV-1 integrase, using a procedure described previously (5). HIV-1 DNA in immunoprecipitates was analyzed by PCR with primers specific either for total viral DNA (*pol*) or for 2-LTR circles. While HIV-1 DNA was detected in the precipitates, no 2-LTR circles could be identified in these samples even after two rounds of PCR with nested primers (Fig. 2A). In a control experiment, 1/10 of the nuclear extract used in the above-described experiment was analyzed without immunoprecipitation and was found to contain 2-LTR circles (Fig. 2B). To exclude artifacts which may be caused by the use of a particular monoclonal antibody in this experiment, immunoprecipitation of nuclear extracts was done with a polyclonal rabbit anti-integrase antiserum. This experiment gave similar results (data not shown). Monoclonal antibody to HIV-1 MA, shown previously to precipitate linear viral DNA (5), also failed to precipitate 1- or 2-LTR circular forms (data not shown). As a negative control, we used monoclonal antibodies to HIV-1 CA, which do not precipitate nuclear preintegration complexes (5). This antibody failed to precipitate both forms of HIV-1 circular DNA as well as linear DNA (data not shown).

According to current models of retroviral integration, integrase is bound to the ends of the viral DNA, providing optimal conformation for the integration (14). Our studies have demonstrated that this association is stable even at high detergent concentrations (5) which cause dissociation of other protein components of the preintegration complex (5, 6). The lack of association of HIV-1 integrase with the 2-LTR circles indicates that integrase does not recognize LTR ends in the context of the 2-LTR junction.

A surprising result of these studies is that viral circular DNAs are not associated with viral proteins that are components of the HIV-1 preintegration complex (5) while they are found within a high-molecular-weight nucleoprotein complex. Since circularization appears to proceed within an intact preintegration complex, at least in vitro (7), it is reasonable to assume that dissociation of the viral proteins was not the cause but the result of viral DNA circularization. Upon dissociation of viral proteins, circular molecules are open for interaction with cellular proteins (i.e., histones), thus maintaining the high-molecular-weight nucleoprotein complex.

Our results are in accord with recent observations by Randolph and Champoux (12), who demonstrated that 2-LTR circular DNAs of simian immunodeficiency virus are enriched for integration-defective molecules. Indeed, DNA molecules with integration-competent ends should be tightly associated with integrase, thus favoring the integration reaction and preventing interaction with cellular enzymes.

Molecules that cannot integrate because of defective ends (12) probably do not bind integrase as tightly as integration-competent molecules. This provides an opportunity for cellular enzymes (DNA polymerase and ligase) to work on the ends of these molecules, resulting in their circularization. Since reverse transcription of the viral genome is accompanied by a high frequency of mutations, some of which result in defective DNA ends (12), there is always a pool of molecules which are substrates for circularization and which do not compete with integration-competent molecules. The formation of high-molecular-weight nucleoprotein complexes of circular viral DNAs and cellular proteins explains the relative stability of these forms (17a) and most likely accounts for their biologic activity.

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