JOURNAL OF VIROLOGY, Aug. 1997, p. 6225–6229 Vol. 71, No. 8
0022-538X/97/$04.00+0

Human Immunodeficiency Virus Type 1 Nucleocapsid Protein Specifically Stimulates Mg^{2+}-Dependent DNA Integration In Vitro

SANDRINE CARTEAU,[1]† SUSAN C. BATSON,[2] LEONORA POLJAK,[2] JEAN-FRANÇOIS MOUSCADET,[1] HUGHES DE ROCQUIGNY,[3] JEAN-LUC DARLIX,[4] BERNARD P. ROQUES,[3] EMMANUEL KÄS,[2] AND CHRISTIAN AUCLAIR[1]*

Laboratoire de Physicochimie et Pharmacologie des Macromolécules Biologiques, CNRS URA 147, Institut Gustave Roussy, 94805 Villejuif,[1] Laboratoire de Biologie Moléculaire Eucaryote, CNRS UPR 9006, 31062 Toulouse Cedex,[2] Département de Pharmacochimie Moléculaire et Structurale, U266 INSERM URA D1500 CNRS, Université René Descartes, 75270 Paris Cedex 06,[3] and LaboRétro, Unité de Virologie Humaine, INSERM U412, Ecole Normale Supérieure de Lyon, 69364 Lyon,[4] France

Received 11 November 1996/Accepted 8 April 1997

The integrase (IN) protein of the human immunodeficiency virus mediates integration of the viral DNA into the cellular genome. In vitro, this reaction can be mimicked by using purified recombinant IN and model DNA substrates. IN mediates two reactions: an endonucleolytic cleavage at each 3′ end of the proviral DNA (terminal cleavage) and the joining of the linear viral DNA to 5′ phosphates in the target DNA (strand transfer). Previous investigators have shown that purified IN requires Mn^{2+} or Mg^{2+} to promote strand transfer in vitro, although Mg^{2+} is the likely metal cofactor in vivo. IN activity in the presence of Mg^{2+} in vitro requires high IN concentrations and low concentrations of salt. Here, we show that the viral nucleocapsid protein NCp7 allows efficient IN-mediated strand transfer in the presence of Mg^{2+} at low enzyme concentrations. This potentiating effect appears to be unique to NCp7, as other small DNA-binding proteins, while capable of stimulating integration in the presence of Mn^{2+}, all failed to stimulate strand transfer in the presence of Mg^{2+}.

A key step in the replication cycle of retroviruses is the integration of a proviral DNA copy of the viral RNA genome into the host cell DNA (1). This step is required for the production of progeny viruses (23) and is mediated by a viral protein, integrase (IN). IN directs two distinct reactions: a terminal cleavage at each 3′ end of the proviral DNA, removing 2 bases (3), and the strand transfer, which results in the joining of processed 3′ ends to 5′ phosphates in the target DNA (3, 12).

The terminal cleavage and strand transfer reactions can be modeled in vitro with purified recombinant IN protein (4). Under simple reaction conditions, purified IN requires Mn^{2+} for efficient activity in vitro, although it most likely utilizes Mg^{2+} as a physiological cofactor. Under standard reaction conditions, with purified IN, little or no strand transfer activity can be detected in the presence of Mg^{2+}. However, enzyme activity can be rescued by high concentrations of dimethyl sulfoxide (DMSO) or polyethylene glycol (11). Mg^{2+}-dependent strand transfer can also be observed in the presence of high concentrations of IN, but this activity is inhibited at physiological salt concentrations (11). In contrast, when IN is isolated from infected cells as part of a preintegrative complex, it can promote efficient integration in vitro by using Mg^{2+} and physiological salt concentrations (2, 9). This discrepancy suggests that one or several components of the preintegrative complex may be required for IN to be active under in vivo conditions (for a review, see reference 27). In an effort to identify potential cofactors, we have attempted to characterize factors that specifically promote IN activity in the presence of Mg^{2+}.

The nucleocapsid protein (NCp7) of human immunodeficiency virus type 1 (HIV-1) has been shown to be a component of nucleoprotein complexes present in the cytoplasm and in the nucleus of infected cells (13). NCp7 binds tightly to RNA and DNA molecules (8, 16, 17; for a review, see reference 6). NCp7 can promote the aggregation of nucleic acids and both the annealing and unwinding of RNA and DNA molecules (16, 17, 21, 25, 28). It is probably through these activities that NCp7 promotes RNA dimerization and encapsidation in vivo (6) and reverse transcription in vitro (21, 22). Results of the experiments presented here demonstrate that NCp7 also exerts strong stimulatory effects on IN activity in vitro. The addition of NCp7 to reaction mixtures containing Mg^{2+} and purified IN greatly stimulates HIV-1 IN-mediated strand transfer in vitro, while other small basic proteins fail to enhance IN activity under the same conditions.

Initial observations and experimental approach. The studies reported here are based on several observations which suggested that NCp7 might promote IN-mediated processing and strand transfer reactions in the presence of magnesium. NCp7 has been shown to stimulate slightly Mn^{2+}-dependent terminal cleavage of 28-bp double-stranded oligonucleotides by IN in vitro (16). This observation prompted us to examine Mg^{2+}-dependent processing of 21-bp oligonucleotides corresponding to the HIV-1 long terminal repeat (LTR) ends in the presence of NCp7. A low yield of cleaved fragments became detectable under these conditions (data not shown). While the use of longer oligonucleotides led to a slight increase in processing (18), we could not detect strand transfer. However, it has been reported that NCp7 can promote the denaturation of short DNA molecules (25). Consequently, we decided to use a

* Corresponding author. Mailing address: CNRS URA 147, Institut Gustave-Roussy, PRII, 39 rue Camille Desmoulins, 94805 Villejuif Cedex, France. Phone: 33 1 42 11 54 48. Fax: 33 1 42 11 52 76. E-mail: auclair@igr.fr.

† Present address: Infectious Disease Laboratory, Salk Institute, La Jolla, CA 92037.

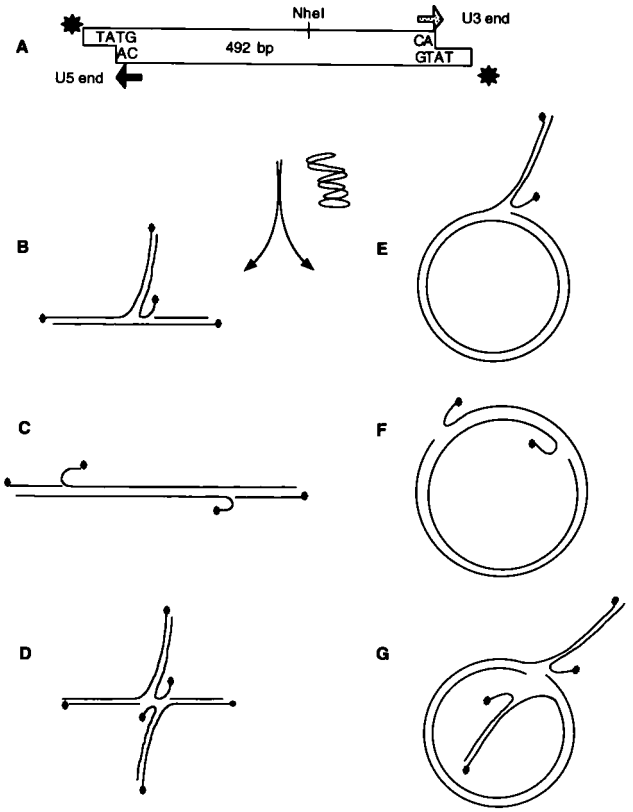

FIG. 1. Integration substrate and products. Plasmid pU3U5 was constructed by cloning a double-stranded oligonucleotide (5′-CCGGGTGTGGAAAATCT CTAGCATATGGAAGGGCTAATTCACTCA-3′; 5′-AGCTTGAGTGAATT AGCCCTTCCATATGCTAGAGATTTTCCACAC-3′), corresponding to the U3 and U5 LTR ends of the HIV-1 cDNA, between the *Ava*I and *Hin*dIII sites of vector pSP65. A second double-stranded oligonucleotide corresponding to the U3 LTR end (5′-ATTGCATATGGAAGGGCTAATTCACTCG-3′; 5′-GCGC CGAGTGAATTAGCCCTTCCATATGCAAT-3′) was then inserted between the *Ssp*I site and the *Kas*I site located at position 2584 of the plasmid. In both oligonucleotides, the underlined bases correspond to the substitution of the GT terminal sequence of HIV-1 DNA introduced in the sequence to produce an *Nde*I restriction site. Digestion of plasmid pU3U5 with *Nde*I produces the 492-bp donor DNA fragment used in our experiments (A). This substrate contains the 20 terminal bp of the HIV-1 5′ and 3′ LTRs at each end, with 2-base 5′ overhangs generated by *Nde*I cleavage that mimic the product of the endonucleolytic cleavage of HIV cDNA by IN. With this substrate DNA and supercoiled pSP65 vector DNA as target, IN-mediated strand transfer reactions can give rise to the following products. The donor can be inserted into another donor molecule, resulting in Y-shaped donor-donor integration products (B), or into a linear fragment of doubled size if a single donor molecule integrates in a concerted fashion (C). Integration can also involve the LTR termini of two or more donor molecules (D), generating DNA molecules of different sizes. Insertion of the donor DNA in the plasmid target will produce a relaxed tagged circular form (E). Concerted insertion of both viral DNA ends of one donor molecule into the plasmid produces a correspondingly larger circular DNA (F). Two or more DNA ends from different donor molecules can also integrate into the plasmid DNA target to generate products of increasing sizes (G). Unique *Nhe*I and *Hin*dIII restriction sites are located in the substrate and in the target DNAs, respectively. Restriction enzyme cleavage and analysis of the products permits detection of products of concerted integration into the plasmid target (see text).

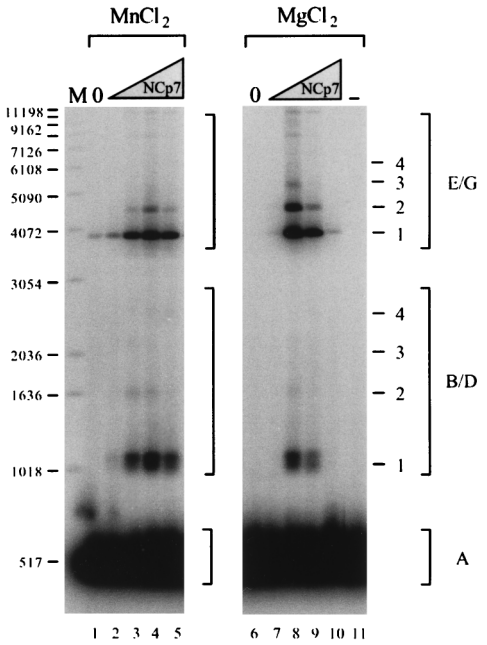

FIG. 2. Stimulation of IN-mediated strand transfer by NCp7. In vitro integration reactions were performed with 10 ng of donor DNA end labeled with ^{32}P by using polynucleotide kinase and [γ-^{32}P]ATP and 25 ng of target DNA, 37.5 nM IN, and increasing concentrations of purified NCp7 in buffer A (20 μM HEPES [pH 7.0], 50 mM NaCl, 10% [wt/vol] glycerol, 2 mM dithiothreitol, 0.1 mg of bovine serum albumin per ml), supplemented with 5 mM $MnCl_2$ (lanes 1 to 5) or 5 mM $MgCl_2$ (lanes 6 to 10). The final DNA concentration was 2.65 μM total DNA base pairs. Recombinant IN was purified as described previously (5, 24). NCp7 was prepared by chemical synthesis and used with both of its zinc fingers fully complemented with zinc (7). All reaction mixtures also contained 0.15 μM Zn^{2+} (final concentration) carried over from protein storage and dilution buffers. All components were preincubated for 30 min at 0°C, and the reaction was allowed to proceed for an additional 30 min at 37°C. NCp7 was added to final concentrations of 135 nM (lanes 2 and 7), 270 nM (lanes 3 and 8), 540 nM (lanes 4 and 9), and 2.7 μM (lanes 5 and 10). Samples in lanes 1 and 6 contained no added NCp7. Lane 11 shows a control sample containing no added IN. Purified DNA samples were electrophoresed alongside molecular weight markers (lane M), and the dried gel was exposed by using a Bas 1000 PhosphorImager (Fuji); the yield of products was determined by using the MacBas program (Fuji). The migration positions of the bands corresponding to free substrate (A), autointegration products (B/D), and tagged circles resulting from integration into target (E/G) are indicated on the right. The labels correspond to those described in the legend to Fig. 1. Numbers to the right of the gels refer to integration of the corresponding number of substrate molecules. The positions of DNA molecular weight markers are shown to the left of the gels (sizes in base pairs).

longer DNA substrate to avoid the possibility that stimulatory effects might be masked by its denaturation. The 492-bp DNA substrate used in our studies, which contains LTR termini at each end to mimic the unintegrated proviral DNA genome, is shown in Fig. 1.

Stimulation of IN-mediated strand transfer by NCp7 with Mn^{2+} or Mg^{2+}. Using this 492-bp substrate, we first asked whether the addition of synthetic NCp7 (7) might stimulate strand transfer in the presence of Mn^{2+}. We incubated the labeled 492-bp donor DNA (10 ng of DNA or 0.75 μM DNA base pairs) with purified recombinant IN (37.5 nM) and target DNA (25 ng of supercoiled pSP65 vector DNA or 1.9 μM DNA base pairs) in the presence of 5 mM $MnCl_2$ and increasing concentrations of NCp7.

As shown in Fig. 2, lanes 1 to 5, the addition of NCp7 in the presence of Mn^{2+} had a marked effect on IN-mediated strand transfer. Under our experimental conditions, integration was stimulated by approximately 16-fold at the optimal concentration of NCp7 used (compare lanes 1 and 4). The efficiency of the reaction, expressed as the percentage of integrated substrate, ranged from 0.5% (lane 1) to 8% (lane 4). The optimal concentration (lane 4) corresponds to a ratio of approximately 1 NCp7 molecule per 5 DNA bp. The addition of a higher NCp7 concentration resulted in inhibition (lane 5). As previously reported, similar stimulatory effects can be observed with other small DNA-binding proteins (4, 20; also see below).

We then carried out a similar experiment in the presence of

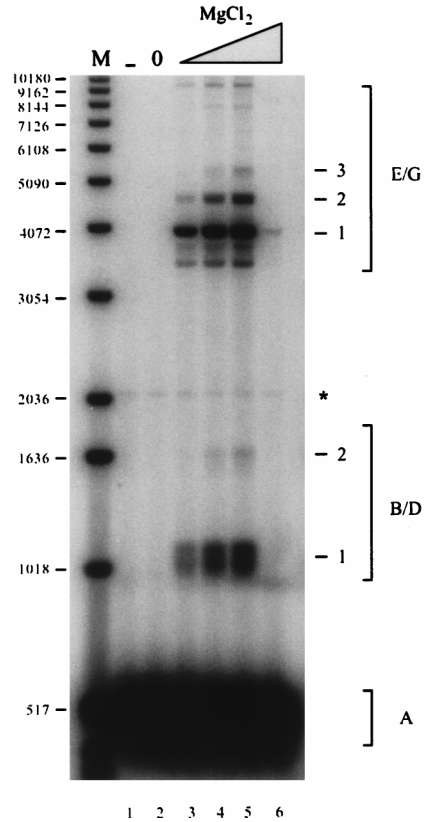

FIG. 3. Effect of increasing concentrations of Mg^{2+} on the strand transfer reaction. Reaction conditions were similar to those described in Fig. 2, except that samples contained a fixed NCp7 concentration of 270 nM. Mg^{2+} concentrations were 1 mM (lane 3), 2.5 mM (lane 4), 5 mM (lane 5), and 10 mM (lane 6). Mg^{2+} was omitted in lane 2, and the sample in lane 1 contained no added IN. Bands are labeled as described in the legend to Fig. 2. The asterisk identifies a minor contaminant in the DNA substrate preparation.

Mg^{2+} (lanes 6 to 10). While no integration was detected in the control sample (lane 6), as expected with the low concentration of IN used in these experiments, the addition of increasing concentrations of NCp7 (lanes 7 to 10) gave rise to efficient strand transfer. Stimulation was maximal (11.5% integration) for the NCp7 concentration corresponding to a ratio of approximately 1 NCp7 molecule per 10 DNA bp (lane 8). We estimate this concentration to be sufficient to coat all DNA molecules (6). Under these conditions, the efficiency of integration was always slightly higher than when Mn^{2+} was used as a cofactor (compare lane 4 to lane 8). Lower NCp7 concentrations (lane 7 and data not shown) had almost no effect, and higher concentrations were inhibitory (lane 10).

Under these conditions, integration was strictly dependent on the presence of Mg^{2+} in the reaction. No strand transfer products were observed in the absence of the cation (Fig. 3, lane 2). The integration efficiency increased with increasing Mg^{2+} concentrations, with a maximum at 5 mM (Fig. 3, lanes 3 to 5). Higher concentrations resulted in inhibition (lane 6). Thus, the stimulation observed in the presence of NCp7 cannot be due to the presence of a contaminating cation such as manganese or zinc in the protein preparation used, since the reaction is dependent on the concentration of magnesium. The mass spectrum of the synthetic NCp7 used also excludes this possibility. Finally, zinc, which could stimulate the Mg^{2+}-dependent activity of IN (19, 29), is present in all reaction buffers (see the legend to Fig. 2) and titration experiments show that Zn^{2+} cannot substitute for manganese or magnesium in the presence or absence of NCp7 (data not shown).

We partially characterized the integration products by digesting purified DNA samples either with *Hin*dIII, which cleaves once in the plasmid target, or with *Nhe*I, which cleaves the donor DNA (Fig. 1A). The different possible products of integration into the plasmid target (Fig. 1, panels E to G) can give rise to characteristic digestion patterns after digestion with either enzyme: for instance, concerted integration into the plasmid target would be expected to produce circular molecules which give rise to 3.5-kb linear molecules after cleavage in either the target DNA or donor DNA. Restriction enzyme analysis showed that, under all experimental conditions examined, tagged circles and Y-shaped molecules were the most abundant integration products (data not shown). We could not detect products of concerted integration.

Distinct stimulatory effects of NCp7 and other small DNA-binding proteins in the presence of Mn^{2+} or Mg^{2+}. It has been previously reported that the addition of small DNA-binding proteins to integration reactions in the presence of Mn^{2+} results in a stimulation of strand transfer activity (4, 20). To determine if the stimulatory effects observed with NCp7 were unique to that protein or could be similarly produced by other small DNA-binding proteins, we added increasing concentrations of purified histone H1 (H1), poly-L-lysine (pL) (4 to 15 kDa), or NCp7 to integration reaction mixtures containing either Mn^{2+} or Mg^{2+}.

In an experiment similar to that shown in Fig. 2, we found that in the presence of Mn^{2+}, all three proteins stimulated integration 8- to 10-fold more than IN alone (Fig. 4A; compare lane 1 with lanes 5, 10, and 15). In this case, the efficiency of the reactions ranged from 1.2% integration in the absence of added protein (lane 1) to 10 to 12% integration (lanes 5, 10, and 15). However, we obtained sharply different results when the same experiment was carried out in the presence of Mg^{2+} (Fig. 4B). The addition of pL or H1 to the reaction mixture had only marginal effects on integration: while we could detect a very low signal (less than 0.02% integration) in the presence of H1 (lane 6), the addition of NCp7 resulted in approximately 10% integration, a 500-fold stimulation (Fig. 4B, lane 14). We performed similar experiments using other basic proteins: HMG-I/Y, HMG-1, and RNase T1 (4, 20) all stimulated integration in the presence of manganese but, as in the case of pL and H1, these proteins also failed to stimulate Mg^{2+}-dependent integration (data not shown).

We next examined this stimulation under conditions under which Mg^{2+}-dependent IN activity is detectable. Several investigators have shown that integration can be detected with IN alone in the presence of Mg^{2+}, when solvents such as DMSO, polyethylene glycol, or dioxane are added to the reaction mixture, by greatly increasing the concentration of IN or by varying the ratio of IN to substrate DNA (4, 11, 14, 20). We therefore repeated the titrations of H1, pL, and NCp7 in the presence of 20% DMSO (Fig. 4C) and in the presence of higher IN concentrations (data not shown). In both cases, we found that all three proteins stimulated integration to markedly different degrees. When HI or pL was added, we detected a 5- to 10-fold stimulation relative to IN alone, corresponding to an increase in the efficiency of the reactions from approximately 0.05% (lane 1) to a maximum of 0.5% (lanes 5 and 9), an effect comparable to that observed in the presence of Mn^{2+} (Fig. 4A). In contrast, stimulation by NCp7 was again much stronger (200-fold [Fig. 4C, compare lanes 1 and 14]), corresponding to approximately 10% integration at the optimal NCp7 concentration. The NCp7 dose response observed in the presence of Mg^{2+} and DMSO is more similar to that seen in

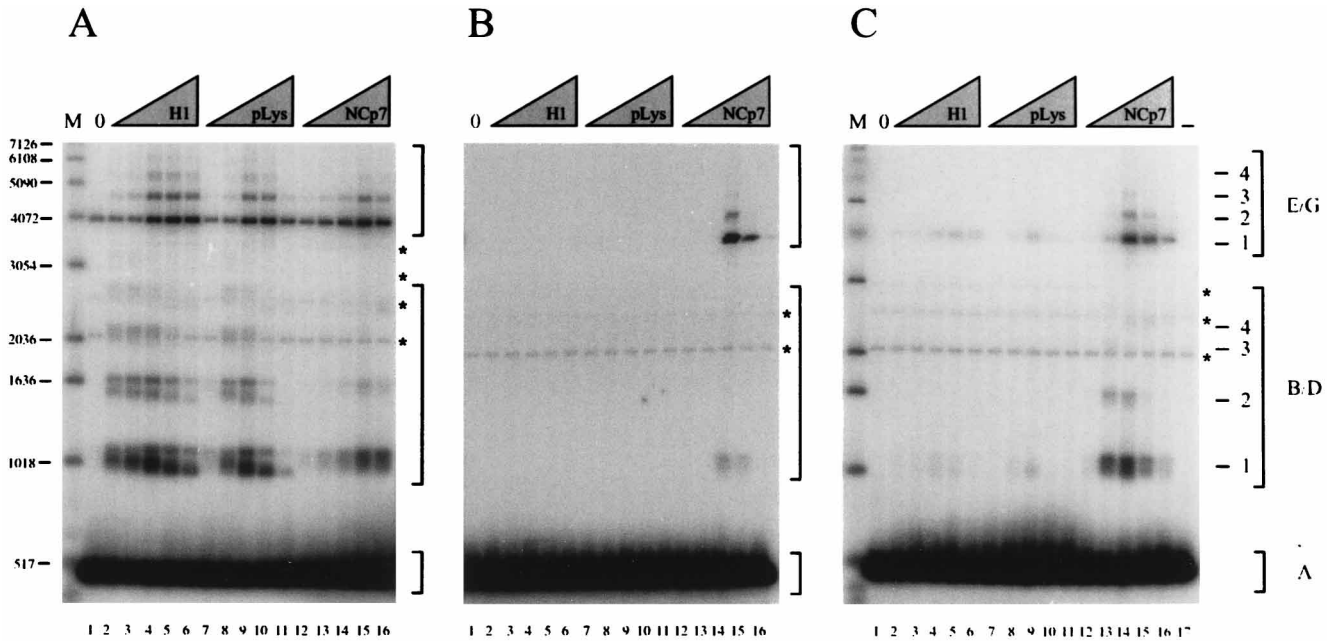

FIG. 4. Comparison of the stimulatory effects of histone H1, pL, and NCp7 in Mn^{2+}, Mg^{2+}, and Mg^{2+} with 20% DMSO. Reactions were performed in the presence of 5 mM Mn^{2+} (A), 5 mM Mg^{2+} (B), or 5 mM Mg^{2+} and 20% DMSO (C). Experimental conditions were identical to those indicated in the legend to Fig. 2. In each panel, lane 1 contained IN alone. In lane 17 of panel C, IN was omitted from the reaction. In all panels, reaction mixtures in lanes 2 through 16 contained IN (37.5 nM) and increasing concentrations of H1, pL, or NCp7. H1 was added to final concentrations of 2.7, 5.3, 10.7, 21.5, and 35 nM (lanes 2 to 6); pL was added to final concentrations of 5.4, 10.5, 27, 54, and 108 nM (lanes 7 to 11); and NCp7 was added to final concentrations of 67.5, 135, 270, 540, and 2.7 μM (lanes 12 to 16). Asterisks identify minor contaminants in the substrate DNA preparation. DNA bands are identified as described in the legend to Fig. 2. Lane M, molecular weight markers (sizes [in base pairs] are indicated to the left).

the presence of Mn^{2+} than in the presence of Mg^{2+} alone (compare lanes 12 to 16 in Fig. 4A to C). However, Mg^{2+}-dependent integration was always highest in the presence of 270 nM NCp7 with or without addition of DMSO (lanes 14 in Fig. 4B and C) while 540 nM NCp7 is the optimal concentration in the presence of Mn^{2+} (Fig. 4A, lane 15). Interestingly, autointegration (products B/D) also appears to be favored at low NCp7/DNA ratios when DMSO is added to the reaction mixture (Fig. 4C, lanes 12 and 13), while the yield of B/D products coincides with that of E/G products in the presence of H1 or pL. We are studying this phenomenon further.

While stimulation of strand transfer by NCp7 is dose dependent when Mn^{2+} is used as a cofactor or when DMSO is present, Mg^{2+}-dependent stimulation exhibits a clear all-or-none response (compare Fig. 4A through C; see also Fig. 2). While no stimulatory effects are observed with low NCp7/DNA ratios under our standard reaction conditions, the addition of an optimal concentration of NCp7 (approximately 1 molecule per 10 DNA bp) results in strong Mg^{2+}-dependent stimulation. However, in experiments using low ratios of NCp7 to total DNA base pairs, preincubation of NCp7 with substrate DNA and IN before addition of target DNA resulted in a marked increase in the yield of strand transfer products (data not shown). In contrast, under identical conditions, preincubation of NCp7 with target DNA and IN before addition of substrate DNA did not give rise to a similar effect. These results suggest that a specific complex is formed between NCp7 and viral LTR DNA sequences during the preincubation period. Taken together, these data strongly suggest that the stimulation of Mg^{2+}-dependent strand transfer activity by NCp7 most likely arises from a specific interaction between the nucleocapsid protein and substrate DNA containing LTR sequences, and perhaps also IN. The latter possibility is currently under investigation.

In summary, our results show that NCp7 can specifically promote efficient Mg^{2+}-dependent IN-mediated strand transfer in vitro, thus restoring a characteristic property of preintegrative complexes isolated from infected cells that is lost in purified recombinant IN. Mg^{2+}-dependent in vitro integration typically requires very high concentrations of purified IN (11), a requirement which is significantly lowered in the presence of an optimal concentration of NCp7. We have tested the stimulation of integration by NCp7 by using IN concentrations ranging from 12.5 to 350 nM. While the stimulation we observe is clearly greatest in the presence of low IN concentrations (37.5 nM in the experiments shown here), higher protein concentrations can promote readily detectable levels of integration under appropriate experimental conditions (4, 11, 20). However, the yield of integration products remains much lower under these conditions: we could detect a maximum of 1 to 2% integration at the highest IN concentration used, while in the presence of an optimal concentration of NCp7, we obtained up to 40% integration by using 150 nM IN. Higher enzyme concentrations inhibited the NCp7-dependent reaction (data not shown). Thus, although the magnitude of the stimulation of integration by NCp7 can clearly be modulated as a function of the enzyme concentration, it is qualitatively specific and independent of the experimental conditions used.

One mechanism whereby NCp7 might stimulate integration is through competition with IN for binding to nonspecific DNA sequences. The displacement of nonspecifically bound IN might increase the concentration of integrase available for binding and acting at the LTR ends of the donor DNA, an interpretation consistent with the observation that other DNA-binding proteins examined uniformly stimulate Mn^{2+}-dependent integration. However, such a simple displacement mechanism does not explain why the stimulation of Mg^{2+}-dependent strand transfer by NCp7 cannot be fully duplicated

by other DNA-binding proteins, even under extreme conditions that support a basal level of IN activity. In the presence of magnesium, NCp7 thus appears to be capable of promoting or stabilizing specific interactions between IN and reactive LTR ends, where it might act to favor the correct oligomerization of IN, which likely acts as a multimer (10, 15, 26). This unique property is consistent with the observation that NCp7 protein is present in viral nucleoprotein complexes (13) and strongly suggests that NCp7 is important for integration in vivo.

S. Carteau and S. M. Batson contributed equally to this work.

We thank Frederic Bushman and Chris Farnet for their help in preparation of the manuscript. We thank Rhône-Poulenc-Rora for their generous gift of integrase.

This work was supported by funds from the Agence Nationale de Recherches sur le Sida (ANRS). Work performed in E. Käs's laboratory was funded by grants from the Centre National de la Recherche Scientifique, the Association pour la Recherche sur le Cancer, the Fondation pour la Recherche Médicale, and the Région Midi-Pyrénées. S. M. Batson and L. Poljak gratefully acknowledge fellowship support from the ANRS.

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