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Cofactors for Human Immunodeficiency Virus Type 1 cDNA Integration In Vitro

Kui Gao,[1] Robert J. Gorelick,[2] Donald G. Johnson,[2] and Frederic Bushman[1]*

Infectious Disease Laboratory, The Salk Institute, La Jolla, California 92037,[1] and AIDS Vaccine Program, SAIC-Frederick, Inc., National Cancer Institute at Frederick, Frederick, Maryland 21702[2]

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We have investigated the function of two DNA binding proteins that stimulate human immunodeficiency virus type 1 cDNA integration in vitro, the cellular HMGa1 protein and the viral nucleocapsid (NC) protein. Of the three forms of NC (NCp7, NCp9, and NCp15), we find that NCp9 is the most effective at increasing integration in vitro; thus, processing of NC may potentially modulate its activities during infection. We also found that maximal stimulation by NCp9 required roughly enough NC to coat the reactant DNAs whereas less HMGa1 was required, and the reactions displayed different optima for divalent metal cofactors and order of addition. These findings reveal probable distinct mechanisms of action in vitro.

Integration of the viral cDNA into a chromosome of the host cell is a required step for retroviral replication (3, 9). Following the completion of reverse transcription, the newly synthesized linear cDNA remains associated with viral proteins, including reverse transcriptase (RT), integrase (IN), matrix (MA), Vpr, and nucleocapsid (NC), and recruits host factors, including HMGa1 [formerly HMG I(Y)] and Ku, to form the preintegration complex (PIC) (2, 11, 12, 24–26). In vitro, the IN protein alone is sufficient to form the initial covalent bonds between the viral cDNA and integration target DNA (4, 5, 10, 19, 29). However, the efficiency of integration in vitro, and correct spatial joining of pairs of viral DNA ends (coupled joining), can be stimulated by protein cofactors such as the cellular HMGa1 protein (1, 11, 17) or the viral NC protein (6, 7). Several further DNA binding proteins have also been proposed to influence integration, including BAF, HMGb1 (formally HMG1), and ini-1 (8, 18, 21–23, 31).

Stimulation of integration by DNA binding proteins in vitro. Here, we report a comparative study of the in vitro roles of HMGa1 and NC derivatives (Fig. 1). In our reactions, a short synthetic oligodeoxynucleotide duplex was used to mimic the viral long terminal repeat (LTR) end and a circular plasmid DNA was used as target. The last 15 bp of the oligonucleotide DNA match the human immunodeficiency virus type 1 (HIV-1) U5 end. The sequence was chosen to match that formed by IN cleavage, which removes two bases from the 3′ end (precleaved substrate). Integration reaction conditions were used that are known to permit coupled joining of the two viral cDNA ends (7, 14, 32–34).

Two main products were detected (Fig. 2A), a linear integration product from coupled joining of two oligonucleotides into the plasmid target (Fig. 2A, left) and a tagged circle product from uncoupled joining of a single oligonucleotide end (Fig. 2A, right). A third product, resulting from integration of one oligonucleotide into another, is also formed but is not visible in the exposures shown.

The conversion of LTR substrate to product is much lower in assays with short LTR oligonucleotides than with longer LTR donors, typically a few percent versus up to 50% (references 7, 17, and 30 and unpublished data). Short LTR oligonucleotides were nevertheless chosen for this study because we wished to investigate methods for improving assembly of integrase-DNA complexes in vitro for potential structural analysis (e.g., reference 13).

LTR DNA sequences were HU534T (5′G TGA CTA ATA AGG GTC TGT GGA AAA TCT CTA GCA) and HU540B (5′ACT GCT AGA GAT TTT CCA CA GAC CCT TAT TAG TCA CGT AC). HMGa1 was prepared as described in reference 11. Wild-type recombinant HIV-1 IN containing a His_6 tag was purified as previously described (13). The strand transfer reaction was carried out in 10 μl of reaction mixture containing 20 mM HEPES (pH 7.3), 5 mM β-mercaptoethanol, 10% dimethyl sulfoxide, 6% polyethylene glycol 8000, 0.1% BSA, 10 mM $MgCl_2$, and 50 mM NaCl (conditions previously reported to support coupled joining of HIV cDNA ends). Typical reactions were carried out as follows: 100 ng of plasmid DNA and 0.2 pmol of substrate DNA were mixed with reaction buffer. Protein cofactor, 13 pmol of HMGa1, or 15 pmol of NC or its derivatives was added, and then 15 pmol of IN was added. The reaction mixture was preincubated for 20 min at room temperature and 30 min on ice (to allow time for assembly of multiple reaction mixtures), and then integration was carried out at 37°C for 60 min. Reactions were stopped by adding 2 μl of stop buffer containing 50 mM EDTA and 1% sodium dodecyl sulfate. One microliter of 1-mg/ml protease K was added. The mixture was incubated at 37°C for 60 min. The sample was analyzed on a 1% Tris-acetate–EDTA agarose gel containing ethidium bromide. Gels were dried and visualized by autoradiography.

A comparison of integration products generated in the reactions containing IN and various additional proteins is shown in Fig. 2B. As reported previously (1, 6, 7, 15, 17, 20), NCp7 and HMGa1 each stimulated integration in vitro (Fig. 2B, lanes 1 and 3) compared to the control with bovine serum

* Corresponding author. Mailing address: Infectious Disease Laboratory, The Salk Institute, 10010 North Torrey Pines Rd., La Jolla, CA 92037. Phone: (858) 453-4100. Fax: (858) 554-0341. E-mail: bushman@salk.edu.

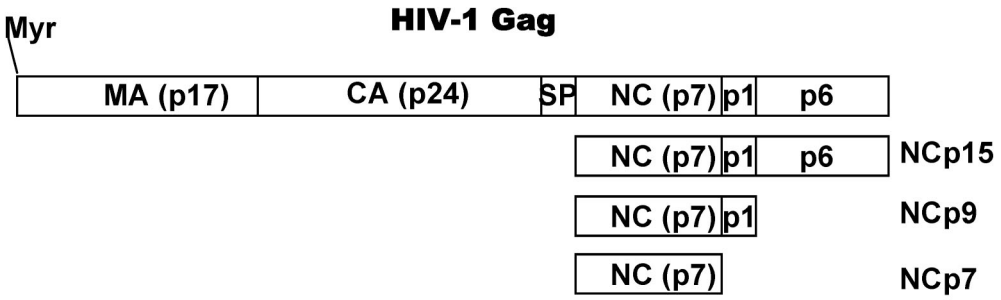

FIG. 1. NC and its derivatives. NCp15 is generated by cleavage of the Pr55 Gag protein. Removing p6 from the carboxyl terminus of NCp15 generates NCp9. Wild-type NCp7 is generated by further removing p1 from NCp9. Overexpression and purification of NC proteins are described elsewhere (data available upon request from R. Gorelick).

albumin (BSA) only, in which integration was undetectable (Fig. 2B, lane 2).

Other protein factors, including the viral proteins RT, Gag, Tat, and Vif and host proteins BAF, Ku, XRCC4, Fen, and poly(ADP-ribose) polymerase were tested in this system as well. Tat and RT showed slight stimulation, while the others showed no stimulation. Several of these proteins were not confirmed to be active in other assays, making these data

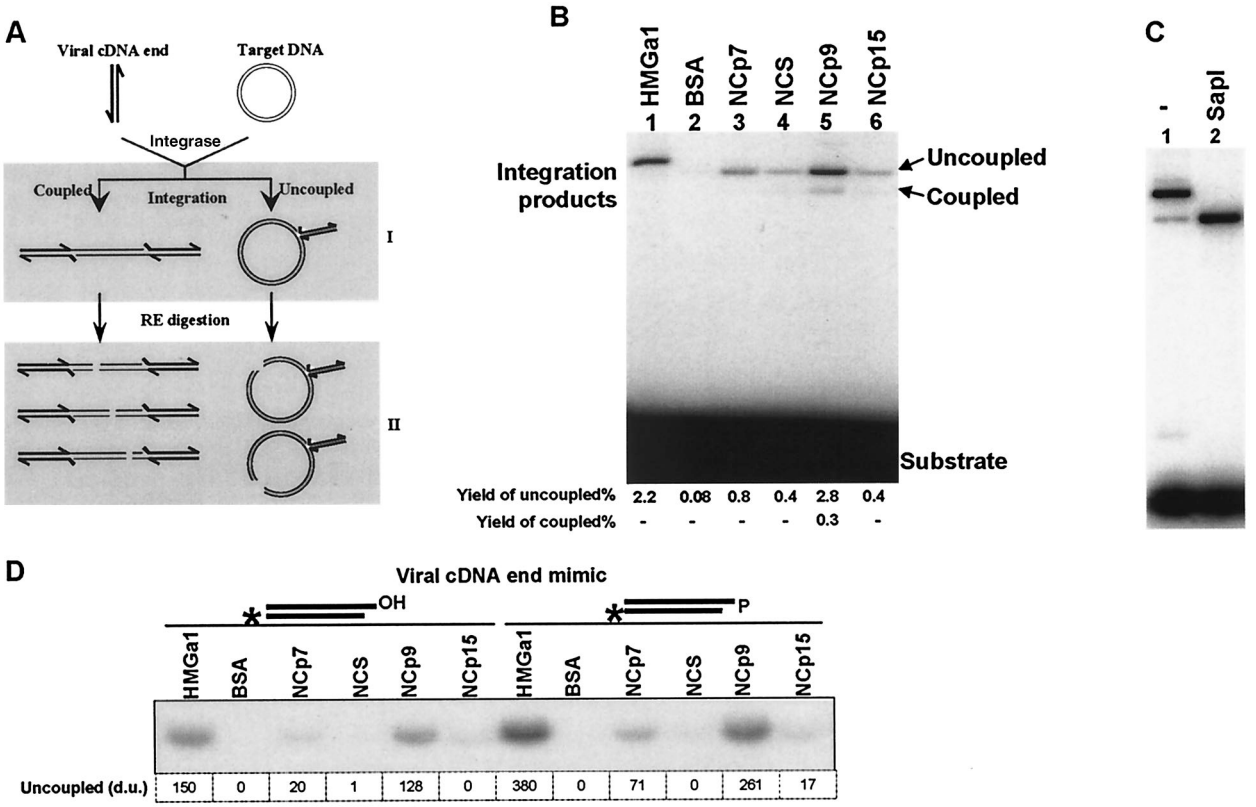

FIG. 2. Stimulation of integration by HMGa1 and NC derivatives. (A) Diagram of integration in vitro and characterization of reaction products. The viral cDNA end was modeled using a 40-bp synthetic DNA duplex. 3′ ends in the viral cDNA are shown as arrows. A plasmid DNA (3,407 bp in length) served as the integration target. The target plasmid DNA contained a unique recognition site for *Sap*I, which was used to characterize the integration products. *Sap*I cleavage of the tagged circle form (right) yields a band the length of the linear plasmid, while cleavage of the coupled joining product yields no single species, due to integration at different points in the target. (B) Stimulation of integration in vitro by HMGa1 (1.3 pmol/μl; lane 1), NCp7 (1.5 pmol/μl; lane 3), NCp9 (1.5 pmol/μl; lane 5), and NCp15 (1.5 pmol/μl; lane 6). BSA (0.8 pmol/μl; lane 2) and the NC zinc finger mutant NCS (1.5 pmol/μl; lane 4) served as controls. Conversion of substrate to product was determined by densitometer analysis. (C) Characterization of integration products. The product of integration in the presence of NCp9 was purified, free oligonucleotide was partially removed, and then the reaction product was treated with *Sap*I (lane 2). An untreated sample is in lane 1. The faint band near the bottom of lane 1 is the product of integration of one LTR into another. (D) Integration is stimulated by the presence of a 5′ phosphate group at the end that becomes joined to target DNA. LTR oligonucleotides containing an unphosphorylated end (left panel) or a phosphorylated end (right panel) are compared in the figure. The relative intensities of bands are quantitated beneath the figure in arbitrary densitometer units (d.u.). See the text for descriptions of the LTR DNA sequences and reaction buffer constituents. Phenol extraction of samples after proteinase K treatment did not affect the results substantially, though without phenol extraction samples with high concentrations of HMGa1 did show slightly lower mobility in the gel.

difficult to interpret, though we note that the BAF preparation was active for reconstitution of salt-stripped PICs and the Fen preparation displayed the expected nuclease activity on oligonucleotide substrates (data not shown).

To investigate stimulation by NC further, a mutant of NC (named NCS [16]) was tested in which all six of the Cys residues involved in zinc binding were replaced with Ser, which eliminated zinc binding. The zinc finger mutation decreased the stimulatory effect by about twofold, comparing NCp7 with NCS (Fig. 2B, lanes 3 and 4). The observation that some stimulation was retained indicated that nonspecific DNA binding partially but not completely accounted for NC stimulation of integration.

We also tested longer forms of NC protein that are products of incomplete proteolytic processing (NCp9 and NCp15 [Fig. 1]) (27, 28). NCp9 stimulated integration to the greatest degree of the three (Fig. 2B, lane 5). The difference in stimulatory activities was also seen in titrations of the three NC derivatives (data not shown). The reaction with NCp9 yielded two products, corresponding to coupled and uncoupled joining products (7, 14, 32–34). Each of the three NC protein preparations displayed about 90% specific activity for DNA binding as measured by fluorescence quenching (M. Urbaneja, J. Casas-Finet, and R. Gorelick, unpublished data).

HMGa1 was slightly less efficient than NCp9 (by about 1.5-fold) and more efficient than NCp7 (by about 3-fold). We note that the stimulatory efficiency was highly dependent on the reaction conditions used (described below).

The structure of integration products was probed by cleavage with restriction enzymes (Fig. 2C), which yielded data consistent with the structures diagrammed in Fig. 2A (see also references 7, 14, 17, and 32 to 34).

The efficiency of integration was found to be increased by the presence of a phosphate residue at the 5′ end of the unjoined strand (Fig. 2D). Substrates with the 5′ phosphate group were more active in reactions with all the cofactors tested (compare the left and right sets of reactions in Fig. 2). During normal infection, the 5′ end of the viral cDNA is thought to be formed by removal of the oligoribonucleotide primer by the viral RNase H enzyme, leaving a 5′ phosphate. In this study, all the LTR substrates used contained the 5′ phosphate.

Response of reactions with HMGa1 or NCp9 to DNA concentration. To investigate the mechanism of stimulation by HMGa1 and NCp9, we titrated various reaction components and compared the effects on integration. Figure 3 shows the effects of altering target DNA concentration in the presence of similar amounts of HMGa1 or NCp9. The target DNA accounted for most of the mass of DNA in the reaction mixture—the LTR oligonucleotides contributed only about 5%. Increasing the concentration of target DNA did not significantly affect stimulation by HMGa1 over the range shown (Fig. 3A, left panel). In contrast, increasing the concentration of target DNA over the same range decreased stimulation by NCp9 quite significantly (Fig. 3A, right panel).

Several further experiments were carried out to establish that the ratio of NCp9 to bulk DNA is a key determinant of the extent of stimulation. Figure 3B presents a titration of target DNA concentration in the presence of 1.5 μM NCp9, showing that optimal stimulation took place at 5 ng of target DNA/μl. Titrations of NC in the presence of a fixed target DNA concentration (Fig. 3C) showed that at 13 ng of target DNA/μl the optimal amount of NC was about 25 ng/μl, while the optimum at 78 ng of target/μl was 75 ng of NC/μl. At all the target DNA concentrations tested, the optimal ratio of NC to DNA base pairs was around 1 mol per 5 to 15 bp (the optimal ratio varied slightly under different reaction conditions).

Figure 3D shows that the extent of coupled joining is influenced by the concentrations of LTR oligonucleotide DNA in the reaction mixture. In particular, reaction mixtures containing relatively high concentrations of the LTR oligonucleotide yielded increased ratios of coupled to uncoupled products (Fig. 3D, compare results with 20 versus 100 fmol/μl). We interpret these data in terms of a model in which NC coats the DNA and competes with IN for binding to nonspecific DNA sites, thereby promoting accumulation of IN at its site of action at the termini of the LTR substrates (see below and references 6, 15, and 20).

Response of reactions with HMGa1 or NC derivatives to metal cofactors. Divalent metal ions, Mg^{2+} or Mn^{2+}, are required as cofactors for integration in vitro. As another means of probing stimulation by HMGa1 and NC in vitro, stimulation was compared in reactions with the two metals. Figure 4A shows that the stimulation by HMGa1 was greater at higher concentrations of $MgCl_2$ (20 mM). In contrast, the optimum for NCp9 was 5 mM, while 20 mM $MgCl_2$ inhibited strongly. Stimulation in the presence of Mg^{2+} and Mn^{2+} was compared in Fig. 4B. The stimulation by HMGa1 (lanes 1 and 7) was much greater in reactions with Mg^{2+} than those with Mn^{2+}. In contrast, stimulation by NC was greater in the presence of Mn^{2+}. In addition, in the Mn^{2+} reactions, the NC derivatives showed less of a difference in their stimulatory effect, whereas the differences were more pronounced in reactions with Mg^{2+}. These results emphasize the differences between stimulation by HMGa1 and NC.

Response of reactions with HMGa1 or NC derivatives to order of addition. The mechanisms of stimulation by NCp9 and HMGa1 were compared further by assaying stimulation under different orders of addition. In Fig. 5, lanes 1 to 6, the DNA substrate and target DNA were mixed with $MgCl_2$ and then the stimulatory protein was added. The mixture was incubated at room temperature for 10 min and then IN was added. In Fig. 5, lanes 7 to 12, the order differed—the protein cofactor and IN were mixed with reaction buffer containing 10 mM $MgCl_2$ and incubated at room temperature for 10 min. The LTR DNA substrate was then added, the mixture was incubated at room temperature for another 10 min, and then target DNA was added.

Preincubation of HMGa1 with the DNAs prior to addition of IN produced the greatest stimulation. With this order of addition, HMGa1 was substantially more efficient at stimulating integration than the NC derivatives. In contrast, in reactions where IN and protein cofactors were premixed, NCp9 was the most efficient stimulatory protein. These findings indicate that HMGa1 and NCp9 differ in their favored pathway.

We note that in a previous report HMGa1 did not stimulate integration under slightly different reaction conditions (7), though several other reports have described stimulation (1, 11, 17). Differences between this work and that of reference 7 include the length of the LTR DNA substrates, the order of addition, and the solution conditions used. Data presented

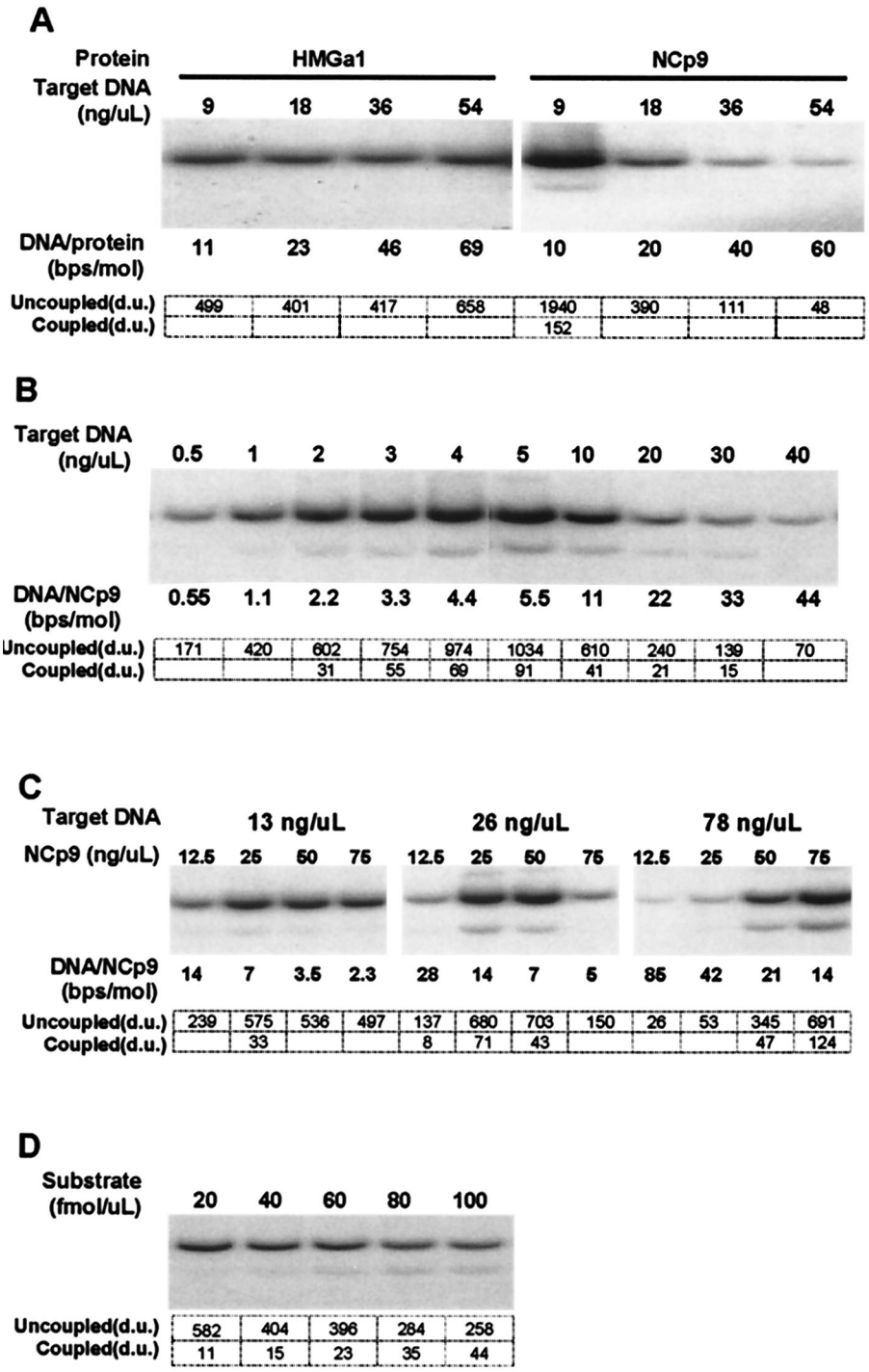

FIG. 3. Distinctive responses of reactions with HMGa1 or NCp9 to target DNA concentration. (A) Titration of target DNA in the presence of 1.3 pmol of HMGa1/μl or 1.5 pmol of NCp9/μl. (B) Titration of target DNA in the presence of 1.5 pmol of NCp9/μl. (C) Titration of NCp9 in the presence of several different concentrations of target DNA. (D) The concentration of LTR DNA affects the ratio of coupled to uncoupled products.

here and in previous studies emphasize that the function of integration cofactors is highly sensitive to the reaction conditions and substrates used.

Conclusions. This study did not achieve the full goal of using protein cofactors to assemble oligonucleotide LTR substrates efficiently into integration-competent particles. NC and HMGa1 did stimulate integration, but most of the oligonucleotide DNAs present remained unreacted. Reactions with longer LTR substrates can yield more efficient conversion of substrate to product (7, 17, 30). However, the studies presented here did reveal several features of the mechanism of stimulation by integration cofactors.

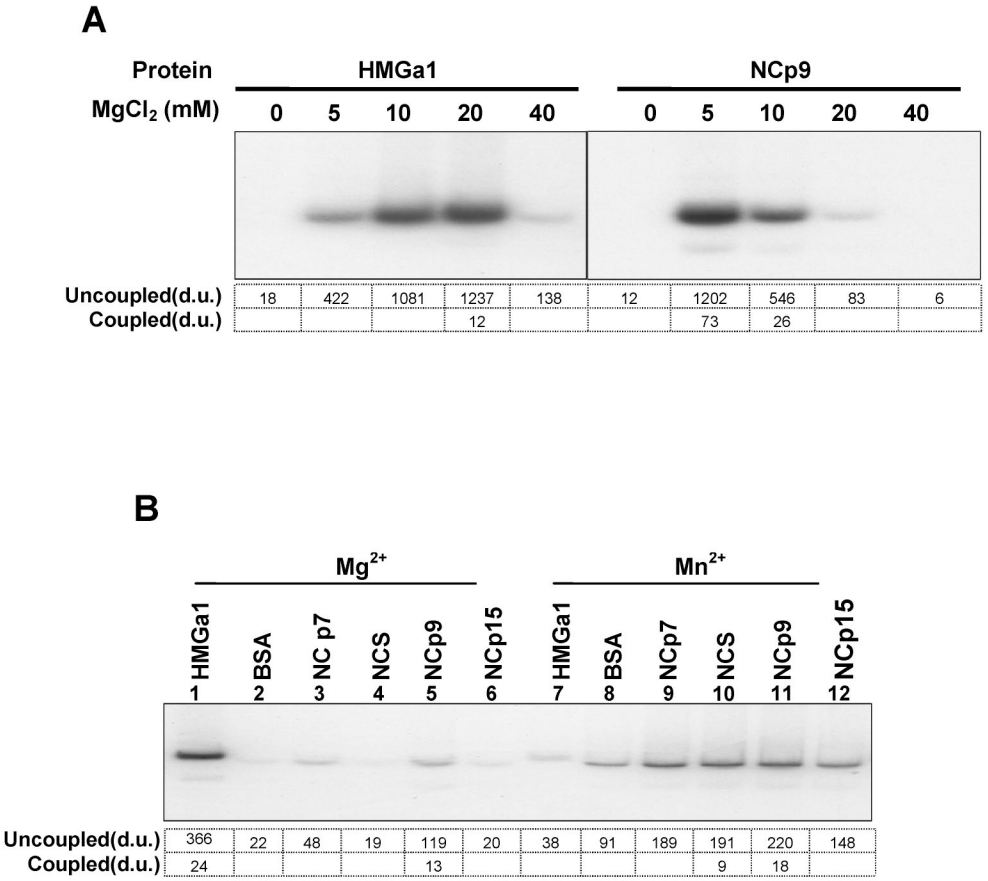

FIG. 4. Differential effects of metal ions in reactions with HMGa1 and NC derivatives. (A) HMGa1 and NCp9 were compared in reaction mixtures containing different amounts of $MgCl_2$ as indicated. (B) The stimulatory effects of HMGa1, NCp7, NCS, NCp9, and NCp15 were compared in the presence of $MgCl_2$ (10 mM) or $MnCl_2$ (10 mM).

NCp9 was found to be a more active stimulatory factor for integration than NCp7 or NCp15. This represents the first demonstration of a role for p1 beyond serving as a spacer region regulating Gag processing (Fig. 1). These findings raise the possibility that processing of NC proteins may regulate the NC activities, potentially coupling viral nucleic acid transactions to NC processing.

We and others have reported that coupled joining can proceed in vitro without added cofactors under some conditions (7, 30), raising the question of whether cofactors are important

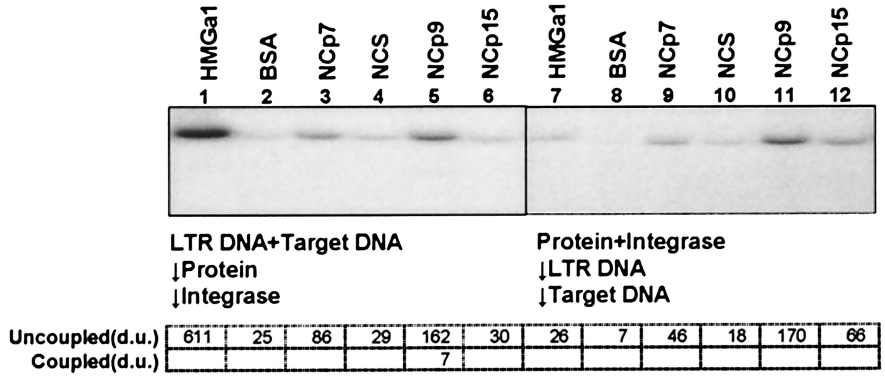

FIG. 5. Differential effects of order of addition on reactions with HMGa1 and NC. In the experiment shown in the left panel, 0.02 pmol of DNA substrate/μl and 10 ng of target DNA/μl were mixed, and then the protein cofactor was added (1.3 pmol of HMGa1/μl, 1.5 pmol of NCp7/μl, 1.5 pmol of NCS/μl, 1.5 pmol of NCp9/μl, or 1.5 pmol of NCp15/μl). IN was added to the mixture last at 1.5pmol/μl. In the experiment shown in the right panel, protein and IN were mixed first, then LTR oligonucleotide was added, and target DNA was added last (concentrations are the same as for the left panel).

in vivo. In our hands, those conditions that do require cofactors are most physiological (5 to 10 mm $MgCl_2$) (7). Evidently, a requirement for cofactors can be bypassed by suitable optimization of in vitro reactions, but we expect that cofactors are nevertheless important during integration in vivo.

We show here that optimal NC stimulation is seen at a ratio of around 1 NC molecule per 5 to 15 bp, potentially enough to coat the reactant DNAs. We favor a model in which NC stimulates integration by competing with IN binding at nonproductive internal sites on the DNA (6, 15, 20). This then helps IN accumulate at high-affinity binding sites at the viral DNA ends. Indirect evidence supports the idea that a similar pathway operates in vivo (1a). HMGa1 apparently stimulates integration in vitro by a different mechanism (11, 25). Unlike NCp9, the stimulation by HMGa1 is not strongly dependent on target DNA concentration, and lesser amounts of HMGa1 are required for optimal stimulation. HMGa1 has multiple DNA binding domains, and HMGa1 stimulation has previously been attributed to condensation of the reactant DNAs (17, 25). We hypothesize that binding of HMGa1 monomers to multiple DNA sites may help fold the reactant DNAs into conformations favoring IN assembly and catalysis.

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