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Efficient Magnesium-Dependent Human Immunodeficiency Virus Type 1 Integrase Activity

ALAN ENGELMAN† AND ROBERT CRAIGIE*

Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland 20892-0560

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The integrase protein from human immunodeficiency virus type 1 (HIV-1) has generally been reported to require Mn^{2+} for efficient in vitro activity. We have reexamined the divalent metal ion requirements of HIV-1 integrase and find that the protein is capable of promoting efficient 3′ processing and DNA strand transfer with either Mn^{2+} or Mg^{2+}. The metal ion preference depended upon the reaction conditions. HIV-1 integrase displayed significantly less nonspecific nuclease activity in reaction mixtures containing Mg^{2+} than it did under the previously described reaction conditions with mixtures containing Mn^{2+}.

An important step in the retroviral life cycle is the establishment of the provirus, an integrated DNA copy of the viral RNA genome. The activities of the virally encoded integrase (IN) protein are necessary for provirus formation (2, 9, 18, 21, 27). IN catalyzes two distinct reactions early during virus infection. In the first reaction, the linear, blunt-ended viral DNA made by reverse transcription is cleaved near each 3′ end at the conserved sequence CA. Following nuclear localization, these processed 3′ ends are covalently joined to the two strands of a chromosomal target site by a pair of DNA strand transfer reactions. The resulting integration intermediate, with unjoined viral 5′ ends and unjoined host DNA 3′ ends, is repaired, most likely by cellular enzymes, to generate the provirus. (See references 16 and 24 for recent reviews of retroviral DNA integration.)

Purified viral IN proteins from a variety of sources possess 3′ processing and DNA strand transfer activities in vitro. These activities, which can be monitored by using double-stranded oligonucleotide DNA substrates that mimic the ends of unintegrated linear viral DNA, require a divalent metal ion cofactor. Efficient 3′ processing and DNA strand transfer activities using oligonucleotide DNA substrates and purified human immunodeficiency virus (HIV) IN have in general been reported to require Mn^{2+} (1, 19, 22, 23), although in one instance comparable activity was reported for reaction mixtures containing either Mg^{2+} or Ca^{2+} (5). In contrast, preintegration complexes isolated from cells after infection can integrate their viral DNA into target DNA in vitro by using either Mn^{2+} or Mg^{2+}, but not Ca^{2+}, as the cofactor (6, 11, 13). We have reexamined the divalent metal ion requirements of recombinant HIV type 1 (HIV-1) IN in the 3′ processing and DNA strand transfer reactions and find that IN can promote efficient catalysis with either Mn^{2+} or Mg^{2+}.

3′ Processing and DNA strand transfer activities. The 3′ processing and DNA strand transfer activities of HIV-1 IN were monitored by standard methods (Fig. 1). The 30-bp U5 viral DNA end was prepared by labeling 150 ng of gel-purified AE144 (5′-TTTTAGTCAGTGTGGAAAATCTCTAGCAGT-3′) with ^{32}P and annealing 150 ng of AE143 (5′-ACTGCTAGAGATTTTCCACACTGACTAAAA-3′), as previously described (3). Labeled double-stranded substrate was separated from unincorporated nucleotides by passage through a Bio-Spin P6 spin column (Bio-Rad Laboratories, Hercules, Calif.) equilibrated with 20 mM NaCl–10 mM Tris-HCl (pH 8.0)–0.1 mM EDTA. Recombinant HIV-1 IN was expressed in *Escherichia coli* and purified from that source as described elsewhere (4, 7). Purified protein (0.33 mg/ml) in 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES [pH 7.5])–1 M NaCl–10% (wt/vol) glycerol–1 mM EDTA–1 mM dithiothreitol (DTT) was concentrated by ultrafiltration with an Amicon Centriprep 10 Concentrator (Amicon, Inc., Beverly, Mass.) under conditions specified by the manufacturer. Concentrated protein was dialyzed in buffer containing 20 mM HEPES (pH 7.5), 1 M NaCl, 20% glycerol, 1 mM EDTA, and 1 mM DTT and centrifuged at 18,500 × *g* for 10 min. Soluble IN (0.91 mg/ml) was divided into aliquots, frozen in liquid nitrogen, and stored at −80°C. Integration reactions (16-μl volumes) were performed at 37°C for 1 h, and aliquots were electrophoresed in 15% polyacrylamide–urea gels as previously described (3). Results were visualized by autoradiography.

In the previously described reaction buffer containing Mn^{2+} (7), the efficiencies of 3′ processing and DNA strand transfer were similar over a range of sodium chloride concentrations, from 24 to 120 mM, when IN was present at 0.64 μM (Fig. 2, lanes 7 to 11). When Mg^{2+} was substituted for Mn^{2+}, 3′ processing and strand transfer products were observed only at the lower end of the range of sodium chloride concentrations tested (Fig. 2, lanes 1 to 6). We had not previously observed this Mg^{2+}-dependent activity because reactions were carried out at higher NaCl and/or lower protein concentrations.

Preliminary experiments (8) revealed that, with 0.64 μM IN and 50 mM NaCl, a 35-bp U5 DNA end supported slightly more Mg^{2+}-dependent activity than did the 21-bp substrate we have previously used (7). Although the difference in activities was less than twofold, 30- or 35-bp substrates were used in the experiments reported here. The concentration of DNA substrate in the Mg^{2+} reaction mixture was varied, and the IN concentration was kept at 0.32 μM (Fig. 3). The 35-bp U5 DNA substrate was prepared by labeling 150 ng of AE349 (5′-GACCCTTTTAGTCAGTGTGGAAAATCTCTAGCGT-3′) at its 5′ end and annealing 150 ng of AE352 (5′-ACTGCTAGAGATTTTCCACACTGACTAAAAGGGTC-3′) as

* Corresponding author. Mailing address: NIDDK, LMB, Building 5, Room 301, 5 Center Dr., MSC 0560, Bethesda, MD 20892-0560. Phone: (301) 496-4081. Fax: (301) 496-0201. Electronic mail address: bobc@helix.nih.gov.

† Present address: Division of Human Retrovirology, Dana Farber Cancer Institute, Boston, MA 02115.

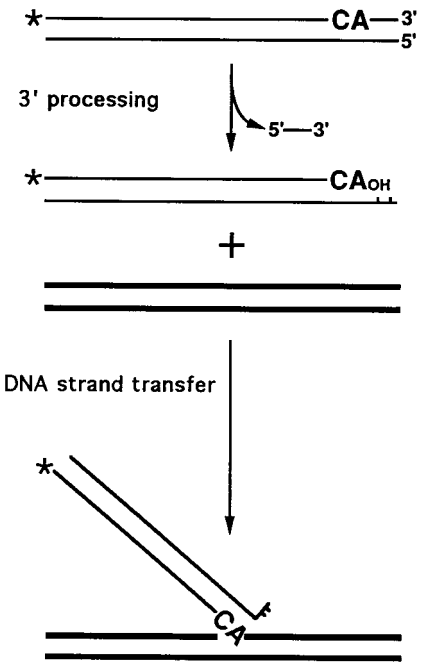

FIG. 1. In vitro activities of retroviral IN. The double-stranded oligonucleotide substrate (thin lines) represents the U5 end of viral DNA following reverse transcription. The substrate is labeled with ^{32}P (marked *) at the 5′ end of the strand which is cleaved by IN. The 3′ processing reaction liberates a dinucleotide cleavage product, yielding a labeled strand 2 bases shorter than the starting substrate. DNA strand transfer of the processed viral end into a target DNA (bold lines) yields a labeled strand which is longer than the starting substrate.

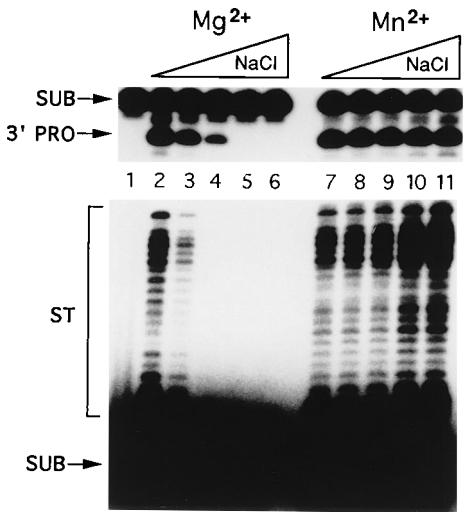

FIG. 2. NaCl dependency of 3′ processing and DNA strand transfer in reaction mixtures containing Mn^{2+} and Mg^{2+}. Reaction mixtures contained 25 nM DNA substrate, 0.1 mg of BSA per ml, 10 mM β-ME, 10% glycerol, 25 mM MOPS (pH 7.2), 0.64 μM IN, various levels of NaCl, and 5.0 mM either $MnCl_2$ or $MgCl_2$. IN was omitted from the reaction mixture in lane 1. Lanes 2 and 7 contained 24 mM NaCl, lanes 3 and 8 contained 48 mM NaCl, lanes 4 and 9 contained 72 mM NaCl, lanes 5 and 10 contained 96 mM NaCl, and lanes 6 and 11 contained 120 mM NaCl. A longer exposure of the gel (lower panel) revealed the population of strand transfer products of different lengths typical of these reactions (3). SUB, substrate DNA; 3′ PRO, 3′ processing product; ST, products of DNA strand transfer.

described above. A larger proportion of substrate was converted to product when the DNA concentration was less than 5 nM. The substrate concentration was therefore fixed at 2.5 nM in subsequent experiments.

With the IN and NaCl concentrations fixed at 0.16 μM and 23 mM, respectively, we tested the effects of various additives on the efficiency of the Mg^{2+}-dependent reaction (Fig. 4). The best conditions included both polyethylene glycol (PEG) and DTT in the reaction mixture (lane 8). A longer exposure of the gel revealed efficient DNA strand transfer activity. Under these conditions, however, little activity was observed when Mn^{2+} was substituted for Mg^{2+} (Fig. 4, compare lanes 7 and 8). Varying the reaction buffer and Mg^{2+} concentration revealed that morpholinepropanesulfonic acid (MOPS) (pH 7.2) and 10 mM $MgCl_2$, respectively, supported optimum 3′ processing and DNA strand transfer (8). The octylphenoxy polyethoxy ethanol (Nonidet P-40) and dimethyl sulfoxide (DMSO) included in these reactions also contributed to the efficiency of the Mg^{2+} reaction, but their influence was less than that of PEG and DTT (8).

Under the reaction conditions which included PEG, DTT, DMSO, and Nonidet P-40, Mg^{2+} was the preferred cation over a 16-fold range of IN concentration (Fig. 5A). Substitution of bovine serum albumin (BSA), glycerol, and β-mercaptoethanol (β-ME) for PEG, DMSO, DTT, and Nonidet P-40 inverted the metal ion preference at lower concentrations of IN (Fig. 5B).

Nonspecific endonuclease activity. We next investigated the nonspecific nuclease activity of IN under Mn^{2+}- and Mg^{2+}-dependent reaction conditions. Supercoiled pBR322 plasmid DNA (a gift of Mary O'Dea, Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases) was incubated with IN in the absence of viral DNA substrate. Reaction mixtures (16 μl) were incubated at 37°C for 1 h, reactions were terminated by the addition of 4 μl of 5× DNA sample buffer (2.5% [wt/vol] sodium dodecyl sulfate, 25% [wt/vol] Ficoll 400, 50 mM EDTA, 0.025% [wt/vol] bromophenol blue), and 10-μl volumes were analyzed in a 0.9% (wt/vol) SeaKem HGT agarose (FMC BioProducts, Rockland, Maine)–TBE (89 mM Tris, 89 mM borate, 2 mM EDTA [pH 8.3]) gel. DNA was visualized by staining the gel with ethidium bromide (0.5 μg/ml).

After incubation of pBR322 with 5 mM EDTA in the absence of IN, the majority of DNA was supercoiled (form I), with a small fraction migrating as nicked circle DNA (form II) (Fig. 6, lane 1). Incubation with either Mn^{2+} (Fig. 6, lane 2) or Mg^{2+} (lane 4) without IN did not reveal any detectable nicking. In contrast, incubation with IN in the presence of Mn^{2+} under the previously reported reaction conditions (7) converted a majority of the supercoiled DNA to the nicked circle form; a significant amount of linear (form III) DNA was also produced (Fig. 6, lane 3). When Mg^{2+} was substituted for

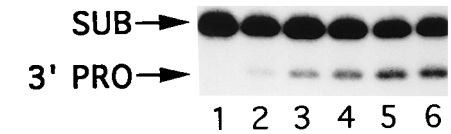

FIG. 3. The level of Mg^{2+}-dependent IN activity is higher at lower concentrations of DNA substrate. Reaction mixtures contained 0.32 μM IN, 0.1 mg of BSA per ml, 10 mM β-ME, 10% glycerol, 25 mM MOPS (pH 7.2), 13 mM NaCl, different concentrations of substrate DNA, and 5.0 mM $MgCl_2$. IN was omitted from the reaction mixture in lane 1. Lane 2 contained 25 nM DNA substrate, lane 3 contained 10 nM, lane 4 contained 5 nM, lane 5 contained 2 nM and lane 6 contained 1 nM. The volume of each reaction mixture analyzed by electrophoresis was adjusted to compensate for the different amounts of DNA substrate in the reaction mixtures. Other labeling is as described in the legend for Fig. 2.

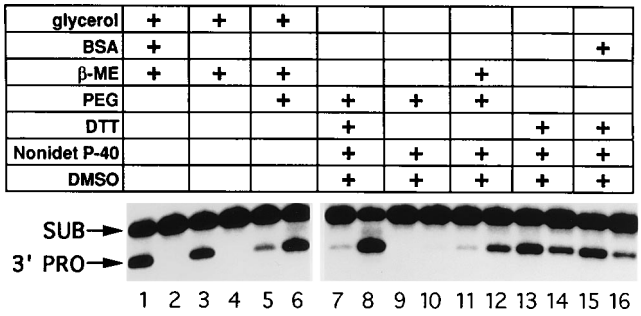

FIG. 4. PEG stimulates Mg^{2+}-dependent activity. Reaction mixtures contained 0.16 μM IN, 25 mM MOPS (pH 7.2), 23 mM NaCl, 2.5 nM of the 30-bp DNA substrate, 7.5 mM either $MgCl_2$ or $MnCl_2$, and various combinations of additives at the following concentrations: glycerol, 10%; BSA, 0.1 mg/ml; β-ME, 10 mM; PEG 8000, 5.0%; DTT, 10 mM; DMSO, 10%; Nonidet P-40, 0.05%. Odd-numbered lanes contained Mn^{2+}, and even-numbered lanes contained Mg^{2+}. Other labeling is as described in the legend for Fig. 2.

Mn^{2+}, substantially less nonspecific nuclease activity was detected (Fig. 6, lane 5), even though similar levels of 3′ processing and DNA strand transfer were observed under these two reaction conditions (compare lanes 2 and 8 of Fig. 5B). Reaction mixtures containing IN, Mg^{2+}, PEG, DMSO, DTT, and Nonidet P-40 revealed more nonspecific activity than did reaction mixtures which contained Mg^{2+}, BSA, glycerol, and β-ME (Fig. 6, compare lanes 6 and 7 with lane 5). However, nicking activity under these conditions was still less than it was under the previously described conditions with Mn^{2+} (Fig. 6, lane 3).

Our results show that recombinant HIV-1 IN can display as efficient 3′ processing and DNA strand transfer activities in reaction mixtures containing Mg^{2+} as it does in reaction mixtures containing Mn^{2+}. Under previously described conditions (7), efficient Mg^{2+}-dependent activity was found to be more sensitive than the corresponding Mn^{2+}-dependent activity to the levels of NaCl (Fig. 2) and IN (Fig. 5B) in the reaction mixtures. These observations may explain previous reports that HIV IN exhibited only partial (5, 22, 23) or no (1) Mg^{2+}-dependent activity in comparison with Mn^{2+}-dependent activity. We were unable to detect either 3′ processing or DNA strand transfer activity in reaction mixtures containing Ca^{2+}

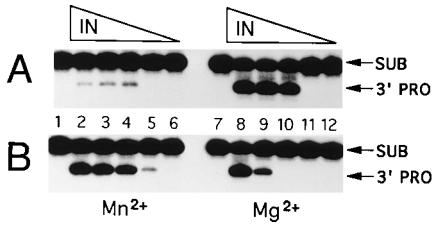

FIG. 5. IN titration under various reaction conditions. (A) Reaction mixtures contained 2.5 nM of the 30-bp DNA substrate, 25 mM MOPS (pH 7.2), 5.0% PEG 8000, 10 mM DTT, 10% DMSO, 0.05% Nonidet P-40, 23 mM NaCl, various concentrations of IN, and 7.5 mM either $MnCl_2$ (lanes 1 to 6) or $MgCl_2$ (lanes 7 to 12). In lanes 1 and 7, IN was omitted from the reaction mixtures; lanes 2 and 8 contained 0.64 μM IN; lanes 3 and 9 contained 0.32 μM; lanes 4 and 10 contained 0.16 μM; lanes 5 and 11 contained 0.08 μM; and lanes 6 and 12 contained 0.04 μM. (B) Reaction mixtures contained the same components as did those shown in panel A, except that PEG, DTT, DMSO, and Nonidet P-40 were replaced by BSA (0.1 mg/ml), β-ME (10 mM), and glycerol (10%). Longer autoradiographic exposures revealed DNA strand transfer products coincident with 3′ processing activity. Other labeling is the same as described in the legend for Fig. 2.

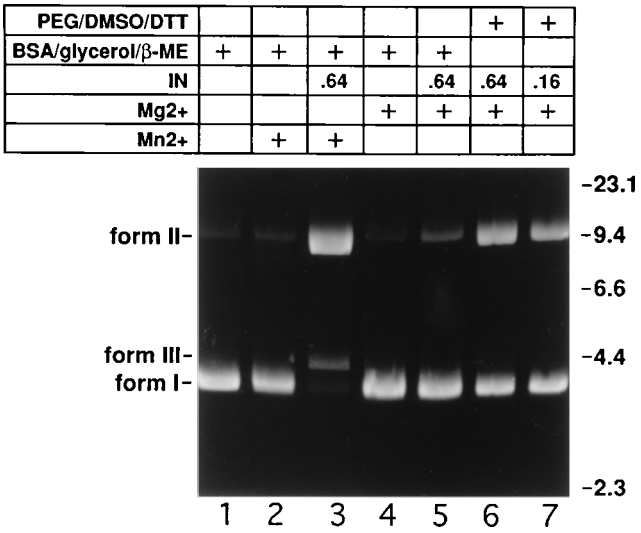

FIG. 6. IN displays more nonspecific nuclease activity in reaction mixtures containing Mn^{2+}. Reaction mixtures in lanes 1 to 5 contained 25 mM MOPS (pH 7.2), 10% glycerol, 10 mM β-ME, 0.1 mg of BSA per ml, 100 ng of pBR322, 23 mM NaCl, and various levels of IN and divalent metal ion. In lane 1, IN was omitted from the reaction mixture, which contained 5 mM EDTA; in lane 2, IN was omitted from the reaction mixture, which contained 10 mM $MnCl_2$; lane 3 contained 0.64 μM IN and 10 mM $MnCl_2$; in lane 4, IN was omitted from the reaction mixture, which contained 10 mM $MgCl_2$; and lane 5 contained 0.64 μM IN and 10 mM $MgCl_2$. Lane 6 contained the same components as did lane 5, except that BSA, glycerol, and β-ME were replaced by PEG 8000 (5.0%), DMSO (10%), DTT (10 mM), and Nonidet P-40 (0.05%). Lane 7 is the same as lane 6 except that the concentration of IN was 0.16 μM. The migration positions of supercoiled (form I), nicked circular (form II), and linear (form III) pBR322 are indicated on the left. The migration positions of molecular mass standards, in kilobase pairs, are indicated on the right.

when we used either blunt-ended or precleaved U5 oligonucleotide DNA substrates.

Unlike activity with reaction mixtures containing Mn^{2+}, striking Mg^{2+}-dependent activity was not observed at lower concentrations of IN (0.16 μM) unless PEG was present (Fig. 4 and 5). The molecular crowding property of PEG (reviewed in reference 28) may increase the effective concentration of IN, and perhaps also that of the DNA substrate. This interpretation is consistent with the observation that PEG was not essential for efficient Mg^{2+}-dependent activity in reaction mixtures containing higher concentrations of IN (Fig. 5B, lane 8).

Our finding that certain reaction conditions enabled HIV-1 IN to display virtually identical 3′ processing and DNA strand transfer activities with either Mg^{2+} or Mn^{2+} (Fig. 5B, lanes 2 and 8) prompted us to evaluate the nonspecific endonuclease activity of IN under these conditions. IN displayed substantially more nonspecific activity with reaction mixtures containing Mn^{2+} than it did in the same reactions with mixtures containing Mg^{2+} (Fig. 6). An active mutant of HIV-1 IN with increased solubility properties, purified following expression in *E. coli* by entirely different chromatographic methods, displayed the same nonspecific nuclease activity as did wild-type IN in mixtures containing the two different divalent metal ions (10). This observation supports the view that the nicking activity reported here is due to IN and not a copurifying, contaminating *E. coli* enzyme. We and others (19) have also observed that many contaminating *E. coli* nucleases are substantially more active in reaction mixtures containing Mg^{2+}.

Mn^{2+} is known to affect the specificities of many Mg^{2+}-dependent enzymes, including polymerases (20, 26) and nucle-

ases (14, 15), which catalyze DNA-dependent reactions. In particular, certain type II restriction endonucleases display less specificity for cleaving their canonical DNA sequence in the presence of Mn^{2+} (15). Mn^{2+}-dependent restriction endonuclease star activity is reminiscent of the nonspecific endonuclease activity of HIV-1 IN reported here. The IN protein from Rous sarcoma virus has previously been shown to exhibit less specificity in the 3′ processing reaction in the presence of Mn^{2+} than in the presence of Mg^{2+} (17). Virion-purified avian myeloblastosis virus IN has also been shown to require Mg^{2+} for the efficient concerted integration of two viral DNA ends into both strands of target DNA (12, 25).

It is not known whether Mg^{2+} or Mn^{2+} is the normal cofactor for HIV IN in vivo. Identification of reaction conditions that support activity with Mg^{2+} enables the enzymology of HIV-1 IN to be studied in vitro with either Mg^{2+} or Mn^{2+} as the cofactor.

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