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A rapid and quantitative assay for inhibition of 3′ cleavage activity of HIV-1 integrase

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Abstract

The human immunodeficiency virus-1 (HIV-1) integrase catalyzes the specific removal of two nucleotides at either 3′ end of each long terminal repeat (LTR) sequence of the proviral DNA duplex. The most commonly used in vitro assays for integrase employ 5′ end ^{32}P-labeled double-stranded oligonucleotides and the product of integrase-associated endonuclease activity is visualized by denaturing gel electrophoresis followed by autoradiography. We report here a simple assay system based upon the liberation of [^{35}S]GT dinucleotide from the 3′ end of a double-stranded U5 LTR sequence derived from HIV-1. The uncleaved labeled substrate and the unlabeled large product are removed by adsorption to polyethyleneimine cellulose followed by centrifugation. The small labeled GT dinucleotide product released in the supernatant is quantitated in terms of counts released as a function of time. Since the method is rapid and quantitative, it should be useful in the kinetic evaluation of inhibitors of the 3′ cleavage activity of HIV-1 integrase. © 1997 Elsevier Science B.V.

Keywords: HIV-1; Integrase inhibition; 3′ cleavage activity assay

1. Introduction

An early event in the replication cycle of human immunodeficiency virus type 1 (HIV-1) is the integration of retroviral DNA into chromosomal DNA of the host cell (Grandgenett and Mumm, 1990; Kulkosky and Skalka, 1990). HIV-1 integrase, a 32 kDa protein, is required for this integration process. It is encoded by the 3′ end of the *pol* gene of HIV-1. Biochemical analysis of

Abbreviations: HIV-1, human immunodeficiency virus type 1; IN, integrase; IMAC, immobilized metal affinity chromatography; PEI, polyethyleneimine; EDTA, ethylenediaminetetraacetate; DTT, dithiothreitol; LTR, long terminal repeat; IPTG, isopropylthio-β-galactoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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recombinant HIV-1 integrase has revealed details regarding the identification of critical residues and functional domains (Drelich et al., 1992; Engleman and Craige, 1992; Bushman et al., 1993; Woerner and Marcus-Sekura, 1993; Lipford et al., 1994). The full length integrase protein contains 288 amino acid residues and can be divided into three domains: the *N*-terminal zinc finger domain (residues 1–49), the catalytic core domain (residues 50–212), and the C-terminal DNA binding domain (residues 220–288).

HIV-1 integrase possesses a highly specific endonuclease activity that cleaves viral substrate DNA at a specific phosphodiester bond 3′ of the conserved dinucleotide (Drelich et al., 1992). This cleavage activity produces the CA_{OH}-3′ end which is incorporated into a target DNA by the strand transfer activity of integrase. The full length integrase protein is required for 3′ processing and DNA strand transfer activities.

Various in vitro assays for the strand transfer and 3′ processing activities of HIV-1 integrase have been described (Craige et al., 1991; Hazuda et al., 1994; Mazumder et al., 1994; Lee and Han, 1996). The most commonly used assays to characterize the 3′ processing activity of recombinant HIV-1 integrase are based on labeling of the 5′ end of an oligonucleotide substrate with ^{32}P. Since the labeled strand is two nucleotides shorter after 3′ processing by HIV-1 integrase, it can be separated from the unreacted labeled substrate by denaturing gel electrophoresis followed by autoradiography (Drelich et al., 1992). However, this method is extremely laborious, requires working with a short half-life isotope, and is difficult to quantitate.

We have developed a more rapid and quantitative solution assay to measure the release of [^{35}S]GT from the plus strand of 20-mer double-stranded DNA corresponding to the HIV-1 U5 LTR sequence. Incubation of this ^{35}S-labeled DNA with recombinant HIV-1 integrase results in a release of the [^{35}S]GT which is a direct measure of 3′ cleavage activity of the enzyme. We also demonstrate the usefulness of this assay in studying inhibition of HIV-1 integrase activity.

2. Materials and methods

2.1. Chemicals

The Fast Flow Chelating Sepharose for immobilized metal affinity chromatography (IMAC) and the pKK223-3 expression vector were from Pharmacia Biotech. SPA Integrase kit and [^{35}S]dATP for sequencing were from Amersham. Oligonucleotides were obtained from Genosys Biotechnologies and National Biosciences and were purified by polyacrylamide gel electrophoresis (PAGE). The Sequenase Version 2.0 kit, and stop solution were from USB. P6 spin columns were from BioRad. Polyethyleneimine (PEI) cellulose and quercetin were from Sigma.

2.2. Recombinant HIV-1 integrase

In order to facilitate the subsequent purification of the HIV-1 integrase protein, a histidine linker was genetically engineered into the *N*-terminus of HIV-1 integrase gene (Drelich et al., 1992) by polymerase chain reaction (PCR). The designed 5′ primer contains the sequence encoding the restriction enzyme *Cla*I site, Pro-Ile-His linker, and the 5′ end of HIV-1 integrase gene. Using the plasmid BH10, which contains the HIV-1 integrase gene as template, the 866 basepair fragment containing the HIV-1 integrase coding region 4263–5129 was generated by 20 cycles of PCR and purified by low melting point agarose gel. The amplified DNA fragment was digested with *Cla*I/*Hin*dIII and subcloned into the prokaryotic expression vector pKK223-3 at the multiple cloning site. Competent *Escherichia coli* strain JM 109 was transformed with the construct and selected for ampicillin resistance. Resulting colonies were analyzed by restriction enzyme analysis and screened for expression of the HIV-1 integrase protein upon induction with 1 mM isopropylthio-β-galactoside (IPTG) at 0.4 $OD_{600\ nm}$ as described elsewhere (Sharma et al., 1991). The 5′ hexa-His linker and HIV-1 integrase sequence of the resulting HIV-1 integrase expression clone, IN5-5, were confirmed by DNA sequencing using Sequenase Version 2.0 sequencing kit and [^{35}S]dATP. The integrase containing clone IN5-5 was grown and

induced with IPTG (Sharma et al., 1991) and the expressed protein was purified by IMAC as described before for HIV-1 reverse transcriptase (Sharma et al., 1991). After purification, the integrase containing fractions were equilibrated to 20 mM HEPES pH 7.5, 10 mM CHAPS, 1 mM EDTA to enhance solubility, and then dialyzed into cold buffer containing 20 mM HEPES pH 7.5, 0.5 M NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol, and 15 mM CHAPS overnight. This was followed by dialysis into 20 mM HEPES pH 7.5, 0.2 M NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, and 15 mM CHAPS for 5 h. Any precipitate present was removed by centrifugation and the purified integrase was stored at −80°C. This preparation of HIV-1 integrase was first characterized with regard to its enzymatic activities (disintegration and strand transfer) and subsequently used for the development of 3′ cleavage assay.

2.3. Gel assay for endonucleolytic cleavage using the LTR oligonucleotide substrate

The 3′ cleavage activity of integrase was assayed by using a double stranded oligonucleotide substrate consisting of the terminal 20 nucleotides of the 3′ U5 LTR end of HIV-1. One oligonucleotide (5′-TGTGGAAAATCTCTAGCAGT-3′) was labeled at the 5′ end with [γ-^{32}P]ATP using T4 polynucleotide kinase. The unincorporated nucleotides were removed by passing the material through a BioRad P6 spin column. The labeled strand was then annealed to 2-fold excess of the complementary oligonucleotide (5′-ACTGCTAGAGATTTTCCACA-3′). For the activity assay, approximately 0.2 pmol of duplex were incubated in a total volume of 10 μl with 600 ng of HIV-1 integrase (1.9 nM) for the indicated time at 37°C in 25 mM Tris pH 8.0, 2 mM $MnCl_2$, 1 mM DTT. The reactions were stopped with the addition of 7 μl of USB stop solution, heat denatured, and 5 μl loaded onto an 8% denaturing polyacrylamide gel. Products were visualized by autoradiography.

2.4. Solution assay procedure for integrase 3′ cleavage activity

A 18/20-mer oligonucleotide duplex consisting of the 3′ U5 LTR end of HIV-1 lacking the two most 3′ nucleotides on the minus strand was used as starting material. Gel-purified oligo IN-2 (5′-TGTGGAAAATCTCTAGCA-3′) (1 μg) was hybridized to 1 μg of gel-purified oligo IN-3 (5′-ACTGCTAGAGATTTTCCACA-3′) in a total volume of 20 μl of 100 mM NaCl. The recessed end was filled using 400 ng of duplex, 200 μCi of dried [^{35}S]dTTP, 200 μCi of dried [^{35}S]dGTP and 1.5 Units Klenow in a total volume of 40 μl labeling buffer (25 mM Tris, pH 7.5, 5 mM $MgCl_2$, 35 mM NaCl, 6.5 mM DTT). The unincorporated nucleotides were removed by passing the material through a BioRad P6 spin column equilibrated in labeling buffer. It was then extracted with phenol–chloroform and used as the substrate for cleavage activity. The specific activity of ^{35}S-labeled U5 LTR substrate was 3.7×10^6 cpm/pmol. For the activity assay, approximately 33 pmol of filled duplex were incubated in a total volume of 10 μl with the indicated amount of integrase for the indicated time at 37°C in 25 mM Tris pH 8.0, 2 mM $MnCl_2$, 1 mM DTT. In reactions containing inhibitors, 1 μl of 10 × inhibitor solution in 50% dimethylsulfoxide was added to the reaction mix. The reaction was stopped with the addition of 200 μl of 50 mM EDTA followed by 700 μl of PEI cellulose (Muller et al., 1993) and vortexed. The samples were agitated for 10 min at room temperature, spun at 14 000 rpm for 10 min in a microcentrifuge and 250 μl of supernatant, containing the cleaved nucleotides, were removed and added to 3 ml of scintillation fluid. The samples were then counted for 1 min on a Packard 1900TR scintillation counter.

3. Results and discussion

3.1. Specificity of recombinant HIV-1 integrase

It is known that full length integrase is required for the 3′ processing activity of HIV-1 integrase

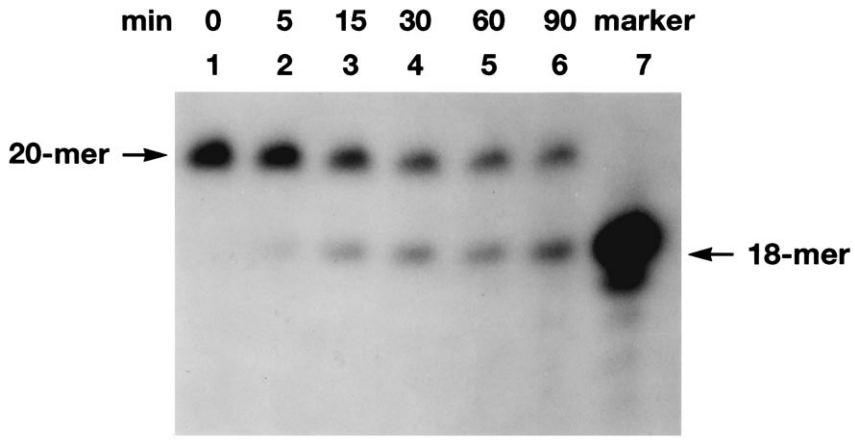

Fig. 1. The 3′ cleavage activity of HIV-1 integrase was assayed using a 5′ end ^{32}P-labeled double-stranded oligonucleotide substrate consisting of the terminal 20 nucleotides of the 3′ U5 LTR end of HIV-1. In the presence of Mn^{2+} integrase cleaves the terminal GT dinucleotide from the labeled 20-mer substrate, producing a labeled 18-mer product. Lanes 1–6 show the time-dependent (0, 5, 15, 30, 60, and 90 min) specific release of the dinucleotide from the LTR substrate separated on an 8% denaturing gel. Lane 7 represents a labeled 18-mer marker.

(Engleman et al., 1994). Recombinant HIV-1 integrase was isolated by IMAC, as described under Section 2. SDS–PAGE of fractions eluted with 300 mM imidazole showed a major protein band that corresponded to a molecular mass of 32 kDa (data not shown). Therefore, we first studied specific cleavage of the 3′ dinucleotide GT from the plus strand of the U5 viral LTR sequence by recombinant HIV-1 integrase using standard gel assay. The expected reaction product after HIV-1 integrase cleavage is shown below:

^{32}P- 5′ TGTGGAAAATCTCTAGCAGT 3′
3′ ACACCTTTTAGAGATCGTCA 5′
U5 LTR
↓ HIV – 1 IN
^{32}P- 5′ TGTGGAAAATCTCTAGCA 3′
3′ ACACCTTTTAGAGATCGTCA 5′

Fig. 1 shows electrophoresis on an 8% denaturing gel, followed by autoradiography. In the presence of HIV-1 integrase and Mn^{2+}, there was time-dependent specific release of the dinucleotide from the LTR substrate. The product of 18 nucleotides was observed, consistent with the known specificity of HIV-1 integrase. The lack of any intermediate products also show specific cleavage of the dinucleotide. These results led us to conclude that our recombinant HIV-1 integrase specifically cleaves a dinucleotide from the 3′-OH and, therefore, is suitable for assay development based on the release of soluble radiolabeled GT from the same substrate.

3.2. Design of the ^{35}S-labeled U5 LTR substrate

The substrate was chosen based on known specificity of HIV-1 integrase (Drelich et al., 1992). To achieve relatively high specific activity, both the nucleotides to be cleaved were labeled. The relatively long half-life of ^{35}S, as opposed to ^{32}P used by others, was the basis for selection of this radioisotope. The radiolabeled substrate and the cleavage product are shown below.

5′ TGTGGAAAATCTCTAGCA [^{35}S] GT 3′
3′ ACACCTTTTAGAGATCGTCA 5′
↓
Release of [^{35}S] GT

3.3. Time- and integrase protein-dependence on [^{35}S]GT production

Fig. 2 shows the time course of [^{35}S]GT generation from the U5 LTR substrate by HIV-1 integrase. Enzyme activity is expressed as counts/min minus a control without HIV-1 integrase. Very little non-specific conversion of this substrate into [^{35}S]GT was observed in the control experiment. As shown, reaction of this substrate under defined assay conditions was linear for at least 20 min. Based on the substrate concentration used, these results show that about 3% of the substrate was hydrolyzed in 20 min at 37°C. The dependence of [^{35}S]GT release on HIV-1 integrase protein concentration showed that GT production was linear between 70 and 500 ng (Fig. 3). Thus, the assay can detect as little as 70 ng of HIV-1 integrase.

3.4. Kinetic analysis

Kinetic parameters for [^{35}S]GT-based substrate (4–90 nM) were obtained by measuring the velocity of [^{35}S]GT production as a function of substrate concentration. The reaction obeyed Michaelis–Menten kinetics when initial rate data

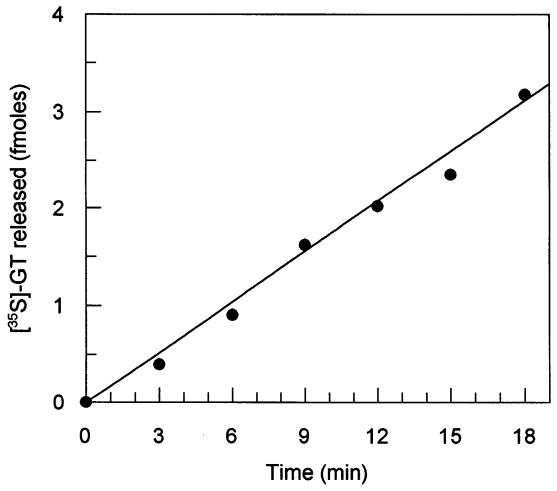

Fig. 2. Integrase 3′ cleavage activity as a function of time. A time course assay was run using 500 ng of HIV-1 integrase (1.5 nM) and 41 nM of the ^{35}S-labeled U5 LTR substrate. Incubation was done at 37°C for the indicated time and the samples processed and counted for the release of [^{35}S]GT dinucleotide. Activity is expressed as counts/min.

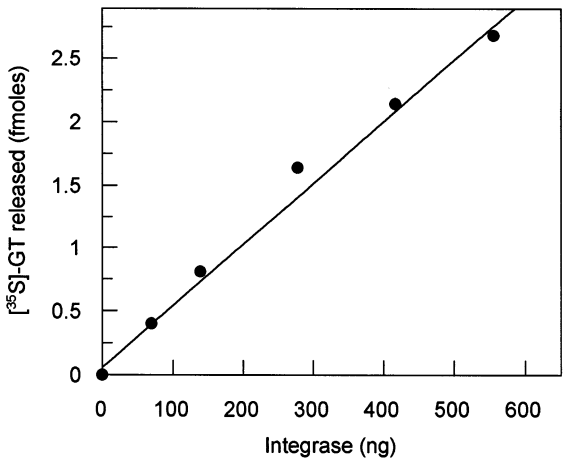

Fig. 3. Integrase 3′ cleavage activity as a function of protein concentration. An enzyme concentration curve was run using HIV-1 integrase (0.2–2 nM) and 41 nM of the ^{35}S-labeled U5 LTR substrate. Incubation was done at 37°C for 15 min using the indicated amount of integrase and the samples were processed and counted for the release of [^{35}S]GT dinucleotide. Activity is expressed as counts/min.

were analyzed using a non-linear least-squares program. The agreement between the experimental points and the theoretical curve calculated from the Michaelis–Menten equation is shown in Fig. 4. We calculate a K_m of 84 ± 10 nM and a V_{max} of 0.42 ± 0.01 fmol/min.

3.5. Inhibition of 3′ cleavage by quercetin

Fig. 5 shows a dose response for inhibition of 3′ cleavage activity by quercetin. About 50% inhibition of 3′ cleavage activity was observed at 9.02 ± 0.24 μM quercetin. An IC_{50} of 23 ± 6 μM for quercetin has been reported in the gel-based 3′ cleavage assay (Fesen et al., 1994). Thus, the results of quercetin inhibition in our solution assay are comparable to that found in the gel assay for inhibition of 3′ cleavage (Fesen et al., 1994). It is concluded that our 3′ cleavage assay should be suitable for determination of IC_{50} of integrase inhibitors.

A number of differences in the activities of HIV-1 integrase in the presence of either Mg^{2+} or Mn^{2+} have been noted (Lee and Han, 1996). The disintegration reaction can be supported by Mn^{2+}, but not by Mg^{2+} (Lee and Han, 1996). A

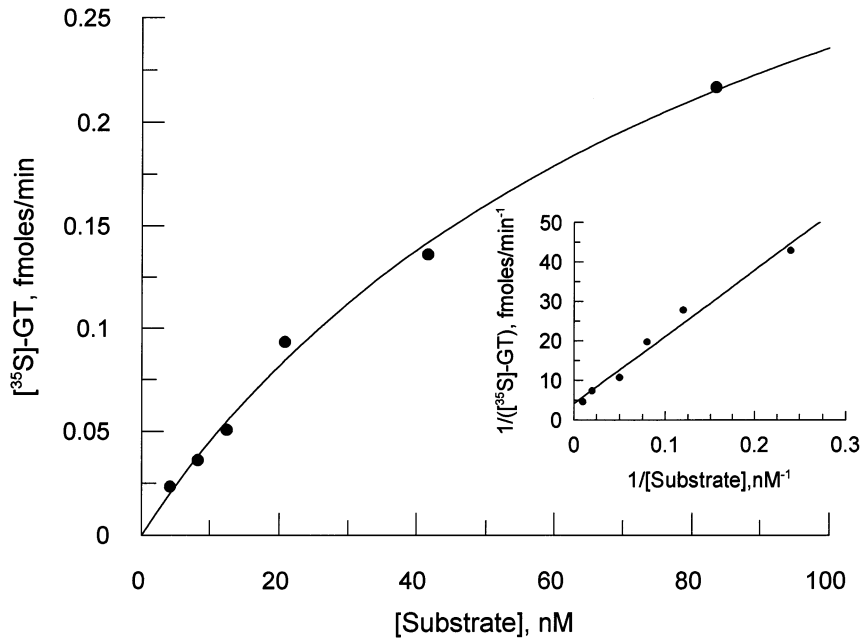

Fig. 4. A Michaelis–Menten plot showing the effect of varying the concentration of ^{35}S-labeled U5 LTR substrate on 3′ cleavage activity of HIV-1 integrase. The assay was done by using 500 ng HIV-1 integrase (1.5 nM) and the indicated amount of substrate at 37°C for 15 min. Velocity was determined by measuring the production of [^{35}S]GT dinucleotide and was expressed as fmol/min. Inset: Lineweaver–Burk plot.

commercial kit from Amersham Life Science is available for large scale screening of inhibitors of the disintegration activity of HIV-1 integrase in the presence of Mn^{2+}. Therefore, to be mutually consistent during secondary evaluation of potential integrase inhibitors, it is desirable to have a quantitative 3′ cleavage assay that works in the presence of Mn^{2+}. No enzymatic activity was observed in the absence of Mn^{2+} or presence of Mg^{2+}. Thus, we chose to develop a 3′ cleavage assay with a short duplex that is known to work in the presence of Mn^{2+} (Lee et al., 1995b). Assays utilizing larger substrates would be required for assessing Mg^{2+}-dependent activity of HIV-1 integrase (Lee et al., 1995a,b).

The development of a quantitative assay for 3′ cleavage activity of HIV-1 integrase was based on previous work on avian myeloblastosis virus (Fitzgerald et al., 1991) and Rous sarcoma virus integrases (Muller et al., 1993). It should be noted that, while this work was in progress, quantitative assays, based on fluorogenic substrates, for 3′ cleavage activity of HIV-1 integrase have ap-

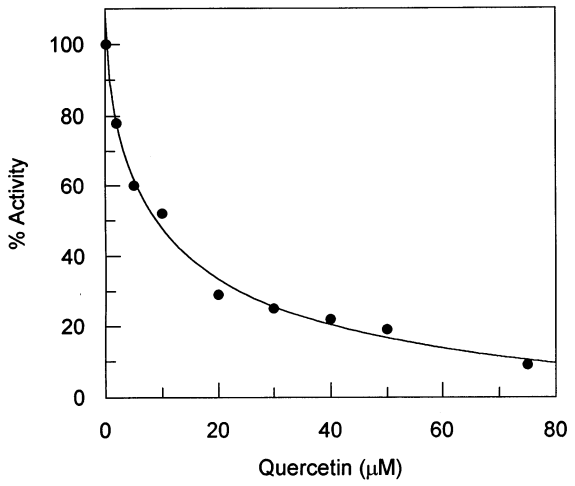

Fig. 5. Dose–response curve for inhibition of 3′ cleavage activity of HIV-1 integrase. Integrase 3′ cleavage assays using ^{35}S-labeled U5 LTR substrate were done in the presence of quercetin using 500 ng integrase, 41 nM ^{35}S-labeled substrate and incubated 37°C for 15 min. Inhibition of activity is expressed as percent activity relative to a non-quercetin containing control.

peared (Hawkins et al., 1995; Lee et al., 1995c). In contrast to HIV-1 reverse transcriptase and HIV-1 protease, for which a large number of specific inhibitors have been documented (Sardana et al., 1992; Balzarini et al., 1993; Saag et al., 1993; Condra et al., 1995; Schinazi et al., 1996), very little is known about selective inhibitors for any of the three enzymatic activities of HIV-1 integrase. The availability of a quantitative 3′ cleavage activity assay should facilitate identification of inhibitors that specifically inhibit 3′ processing activity of HIV-1 integrase.

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