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Characterization of Recombinant Integrase of Human Immunodeficiency Virus Type 1 (Isolate Bru)

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Abstract—Integration of the human immunodeficiency virus type 1 (HIV-1) DNA into the human genome requires the virus-encoded integrase protein. The recombinant integrase protein of HIV-1 (isolate Bru) was prepared by constructing a plasmid based on pET-15b encoding the integrase gene. Integrase of HIV-1 was purified using a bacterial expression system (*Escherichia coli*). The main kinetic parameters of HIV-1 integrase ($K_m = (3.7 \pm 0.2)\cdot10^{-10}$ M, $k_{cat} = (1.2 \pm 0.3)\cdot10^{-7}$ sec^{-1}) were determined using an oligonucleotide duplex constructed on the basis of the U5-terminal sequence of proviral HIV-1 DNA as the substrate. Inhibition of integrase by aurintricarbonic acid ($[I]_{50} = 6.3 \pm 0.4$ μM) and dependence of integrase activity on Mg^{2+} and Mn^{2+} concentration were studied.

Key words: integrase, human immunodeficiency virus type 1, isolate Bru, aurintricarbonic acid

Inhibitors of reverse transcriptase and HIV proteinase are now used in treatment of human immunodeficiency. However, preparations based on these inhibitors are toxic for humans due to homology of viral proteinase and eucaryotic proteinases, and reverse transcriptase and cell DNA polymerases, respectively. In comparison with all other enzymes, a multifunctional HIV-1 integrase is considered the most promising target for production of anti-retroviral preparations [1].

Integration of proviral DNA into the host-cell genome is a necessary step in the life cycle of human immunodeficiency virus type 1 (HIV-1) and requires the virus-encoded integrase protein [2]. The integrase catalyzes two reactions resulting in incorporation of the viral genome into the host-cell DNA. During the 3′-processing reaction, a linear proviral DNA synthesized in the reverse transcription reaction is hydrolyzed at each 3′-end via a conservative CA dinucleotide, and a GT dinucleotide is thus removed. The next integration step proceeds in the nucleus and includes formation of single-strand breaks in the host DNA and ligation with the processed 3′-OH-ends of proviral DNA [3].

Retroviral integrase (28-34 kD) is encoded in the *pol* gene as a single Pol polypeptide and cut by a viral proteinase. The tetramer is an active form of the enzyme, but dimer is also shown to exhibit catalytic activity [4]. According to NMR data, the protein consists of three domains: N-terminal, central (or catalytic), and C-terminal [4, 5].

The N-terminal domain contains a conservative His-His-Cys-Cys motif (HHCC motif) that binds Zn^{2+}; this is supposed to enhance integrase multimerization and to increase the enzyme activity [6]. The catalytic site of the enzyme includes three amino acids: Asp64, Asp116, and Glu152 necessary for exhibiting catalytic activity [7]. These amino acid residues coordinate Mg^{2+} and Mn^{2+} and from D,D-35 E-motif which is the only conservative one among retroviral integrases, retrotransposons, and some bacterial transposases [8]. The C-terminal domain is responsible for nonspecific DNA binding.

As shown recently, the nucleotide sequence and functional activity of integrase in HIV-infected cell culture markedly vary among virus isolates [9]. Now recombinant integrase is obtained by recloning from highly productive molecular clone of HIV-1 pNL4-3 [10-12]. The sequence of the *pol* gene encoding integrase in pNL4-3 was homologous to DNA NY5 isolate [13].

Results of inhibitory analysis *in vitro* and *in vivo* using integrase from pNL4-3 poorly correlate [1]. They can possibly differ due to the ways of estimation of integrase activity as well as the difference in nucleotide sequence of the gene encoding the enzyme from various HIV-1 strains. Thus, for production of efficient antiretroviral preparations based on integrase inhibitors, it is worthwhile to use a set of recombinant proteins corresponding to various virus strains.

To study kinetic parameters of integrase, we obtained a construction based on HIV-1 (isolate Bru) for integrase expression. HIV-1 Bru is widely used for analysis of

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potential inhibitors of HIV-1 replication in infected cell culture [14].

MATERIALS AND METHODS

Components of the bacterial media were from Difco (USA); chromatography sorbents from LKB-Pharmacia (Sweden); dNTP and aurintricarbonic acid from Sigma (USA); *Bam*HI and *Nde*I restriction endonucleases, Taq-DNA polymerase, T4 DNA ligase, and the Klenow's fragment of *Escherichia coli* DNA polymerase I from Sibenzyme (Russia). Oligonucleotides were synthesized by Dr. Yu. A. Gorbunov (Vector, Kol'tsovo).

Isolation of HIV-1 DNA. The total DNA from culture of the infected MT-4 lymphoid cell was isolated according to the standard procedure [15] on the fourth day after infection by HIV-1 (isolate Bru) (infectious titer-30).

Cloning of HIV-1 integrase. The HIV-1 DNA fragment containing a nucleotide sequence encoding integrase was amplified by polymerase chain reaction according to the standard procedure [16] by two pairs of primers:

first pair
In1-fwd 5′-TTCAAGCACAACCAGATAAA-3′,
In1-rev 5′-ACCAATCTAGCATCCCCTAG-3′,

second pair
In2-fwd 5′-CCAGTG***CATATG***GCTGGAATCAGGA-AGGTA-3′,
In2-rev 5′-GGTCCT***GGATCC***TCATCCTGTC-TACTTG-3′.

In the first step, there was produced a fragment of 150 bp on both ends longer than the nucleotide sequence encoding integrase. Primers In2-fwd and In2-rev contained *Nde*I and *Bam*HI reconstruction sites (highlighted in bold), respectively, necessary for the subsequent gene cloning into the expressing vector. The amplified DNA fragments were isolated from polyacrylamide gel by electroelution. The DNA fragment containing integrase was cloned into the pET-15b plasmid vector from Novagen (USA) containing the T7 phage promotor via the *Nde*I and *Bam*HI sites. This construction allows production of the protein with additional amino acid sequence at the N-end—six His residues for possible purification on affinity sorbent. Clone sampling and isolation of plasmid DNA were performed according to the standard procedure [15].

Isolation and purification of recombinant integrase. For integrase expression, *E. coli* BL21 (DE3) cells containing the RNA polymerase gene of T7 phage were used [17]. Cells transformed by plasmid pET-15b-IN (Bru)B1 were grown at intensive aeration on LB medium (250 ml) with ampicillin (200 μg/ml) at 37°C to A_{600} = 0.6-0.8. Then transcription of the gene encoding integrase was induced by addition of isopropyl thiogalactoside to the final concentration 1 mM and the reaction mixture was incubated for 3 h at 37°C. Cells were precipitated by centrifugation, resuspended in 10 ml of buffer A containing 20 mM Hepes, pH 7.5, 1 M NaCl, 1 mM mercaptoethanol, and 10% glycerol and ultrasonicated (22 kHz, 3 times for 20 sec, 0°C). Ultrasonicated lysate was placed in an ice bath for 30 min. Cell fragments were removed by centrifugation (14,000*g*, 20 min) and the supernatant was applied onto a column with Ni-NTA agarose (1 ml) equilibrated with the same buffer. Protein fractions were eluted with a linear gradient of imidazole concentration (0-800 mM, 2 × 10 ml) in buffer A. Fractions containing electrophoretically homogeneous protein with molecular mass 32 kD were pooled and dialyzed twice for 8 h against buffer containing 25 mM Hepes, pH 7.5, 0.5 M NaCl, 1 mM mercaptoethanol, and 40% glycerol. The enzyme was stored at −20°C.

Purity of protein preparations was analyzed by SDS-PAGE [18]. The nucleotide sequence encoding integrase was determined according to Sanger [19] using a T7 Sequenase version 2.0 sequence kit from Amersham (USA). Homology of the studied nucleotide sequence and the sequence of *pol* gene encoding integrase of NY5 isolate chosen from Gene Bank HIV Sequence Database (Los Alamos National Laboratory, USA) was checked using the BLAST program.

Estimation of integrase activity. Integrase activity was estimated during the processing reaction using the 3′-labeled specific substrate of integrase. A 21-bp duplex constructed on the base of the U5-terminal sequence of the proviral DNA HIV-1 was used as the substrate [20]. Synthetic oligodeoxyribonucleotide 19 nucleotides in length 5′-GTGTGGAAAATCTCTAGCA-3′ was hybridized with that 21 nucleotides in length 5′-ACTGCTA-GAGATTTTCCACAC-3′ and then construction was completed using [α-^{32}P]dGTP (Institute of Biochemistry, Siberian Branch of the Russian Academy of Sciences) and TTP using the Klenow fragment of *E. coli* DNA polymerase I [15]. The preparation was purified by SDS-PAGE with subsequent electroelution.

Integrase activity was assayed by excision of the radioactively labeled GT dinucleotide from the 3′-end of one of the strains of the specific DNA duplex. The reaction mixture (45-50 μl) contained 10 mM Hepes, pH 7.5, 5 mM dithiothreitol, 10 mM $MgCl_2$, 80 mM NaCl, 10% polyethylene glycol 6000 (PEG-6000), and 70 ng of integrase. During determination of kinetic reaction parameters (K_m, k_{cat}), concentration of the radioactively labeled duplex was 10^{-11}-10^{-6} M. The reaction mixture was placed in an ice bath for 10 min and then incubated for 1 h at 37°C. The reaction products were analyzed by electrophoresis in 20% polyacrylamide gel under denaturing conditions (7.5 M urea) with subsequent autoradiography. For quantitative analysis of the reaction products, the gel fragments corresponding to the products on

autoradiograph were cut out and radioactivity was measured using a Mark-III counter from Searle Analytical Research (USA).

For the specific substrate, K_m and k_{cat} were determined from the Michaelis–Menten equation using the Enzfitter program from Elsevier Biosoft (The Netherlands).

To determine ion concentrations at which the enzyme activity was maximal, $MnCl_2$ and $MgCl_2$ concentrations were varied. Along with the abovementioned standard components, the reaction mixtures contained 8 nM radioactively labeled duplex.

During determination of aurintricarbonic acid concentration corresponding to the 50% relative enzyme activity ($[I]_{50}$), the reaction mixture contained the abovementioned standard components, the analyzed compound at various concentrations (10^{-7}-10^{-3} M), and 8 nM radioactively labeled duplex.

All calculations were performed using the linear portions of kinetic curves. The uncertainty in the constant determination did not exceed 10-30%.

RESULTS AND DISCUSSION

The construction scheme for expression of HIV-1 integrase is presented in Fig. 1. To increase specificity of polymerase chain reaction (PCR) and amount of the amplified product, amplification was performed in two steps. For analysis of nucleotide sequence of HIV-1 (isolate Bru) [21], two pairs of specific primers were chosen: In1-fwd–In1-rev and In2-fwd–In2-rev. A two-step poly-

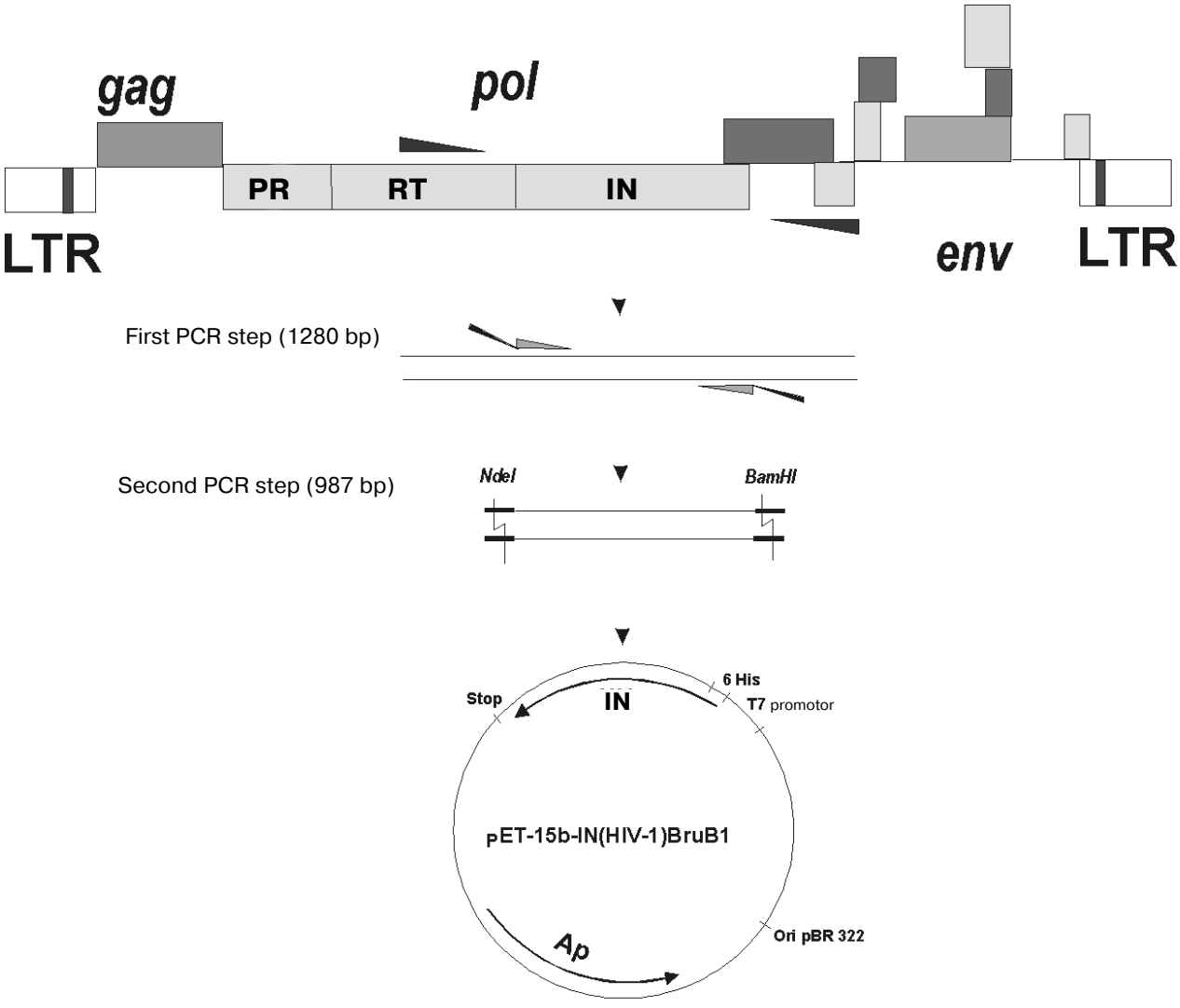

Fig. 1. Scheme of construction of the pET-15b-IN (HIV-1)BruB1 plasmid for expression of recombinant HIV-1 integrase. PR) HIV proteinase; RT) HIV reverse transcriptase; IN) HIV integrase; LTR) long terminal repeat; *gag*, *pol*, *env*) HIV genes.

merase chain reaction provided the necessary amount of DNA fragment encoding integrase with the defined terminal sequences (Fig. 2) for cloning into expressing pET-15b vector via *Nde*I-*Bam*HI sites.

SDS-PAGE of the total protein of cell lysate (Fig. 3, lanes *1*, *2*) indicates that after induction during 3 h, expression of integrase in *E. coli* BL21 (DE3) cells is produced in an amount significantly exceeding the expression level of each of the cell proteins. Affinity chromatography on Ni-NTA agarose with elution with a linear gradient of imidazole concentration allowed isolation of SDS-PAGE-homogeneous integrase (Fig. 3, lanes *4-6*).

Catalytic activity of the recombinant integrase was studied using the 3′-processing reaction [20]. Enzyme activity was assayed via excision of the radioactively labeled product (GT) from the specific substrate. For specific duplex, K_m was $(3.7 \pm 0.2)\cdot10^{-10}$ M, and k_{cat} of the 3′-processing reaction with this substrate was $(1.2 \pm 0.3)\cdot10^{-7}$ sec^{-1}.

It should be noted that for the same specific substrate in the 3′-processing reaction catalyzed by recombinant integrase based on pNL4-3, K_m by one order of magnitude differs from that obtained by us—$(1.5\text{-}3.5)\cdot10^{-9}$ M [22, 23].

Comparing the nucleotide sequence of recombinant integrase (Bru) obtained by us with that of pNL4-3 integrase (Fig. 4), we detected nucleotide replacements resulting in the following changes in the amino acid residues: Glu(10)Asp, Val(113)Ile, Ile(151)Val, and Val(244)Leu. It should be noted that Glu(10)Asp replacement occurs in the HHCC-fragment of the N-terminal domain, and Val(113)Ile and Ile(151)Val replacements occur in the central domain in the nearest neighborhood of amino acid residues of the catalytic site—Asp116 and Glu152.

Using site-directed mutagenesis, Esposito and coauthors [24] showed that the specific CA dinucleotide is bound near Glu152 included in the active site mainly via Tyr143 and Gln148. Integrase catalytic activity decreases on replacements Val(113)Lys and Ile(151)Ala [25].

Amino acid sequences of Bru and pNL4-3 integrases differ in the same positions, but the amino acid replacements do not cause change in the protein charge or solubility. Nonetheless, our data indicate that such difference in HIV-1 (Bru) integrase results in increase in affinity of the specific substrate.

The catalytic activity of HIV-1 integrase depends on the presence of cofactors—Mn^{2+} or Mg^{2+}. It is known that bivalent metal cations induce conformational changes in the structure of integrase [7]. Enzyme activity also markedly varies depending on the reaction conditions: NaCl concentration, buffer composition, stabilizer used (glycerol, PEG, NP-40) [7, 26]. Since optimal concentrations of Mn^{2+} and Mg^{2+} cofactors in the 3′-processing reactions catalyzed by HIV-1 integrase significantly depend on the buffer composition, it was necessary to determine these parameters.

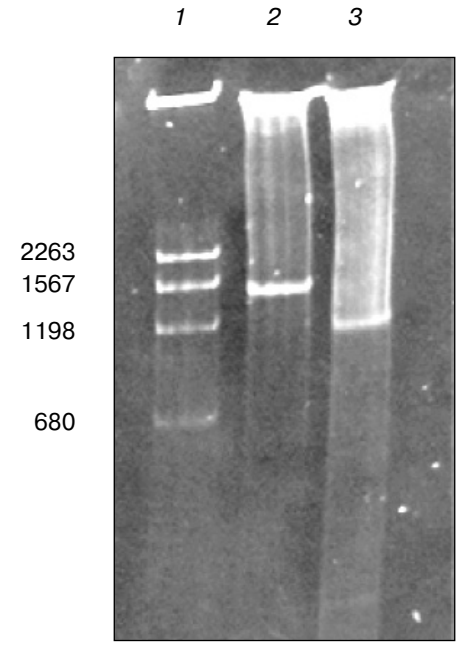

Fig. 2. Amplification of the HIV-1 (isolate Bru) *pol* gene fragment encoding integrase: *1*) markers (pET/RsaI) (molecular mass in bp is given at the left); *2*, *3*) the first and the second PCR steps, respectively.

For Mg^{2+}, the optimal concentration for the maximal enzyme activity is 2 mM less than for Mn^{2+} (Fig. 5). It should be noted that on further increase in Mn^{2+} concentration integrase activity decreases. Decrease in the enzyme activity in the presence of Mn^{2+} can be probably

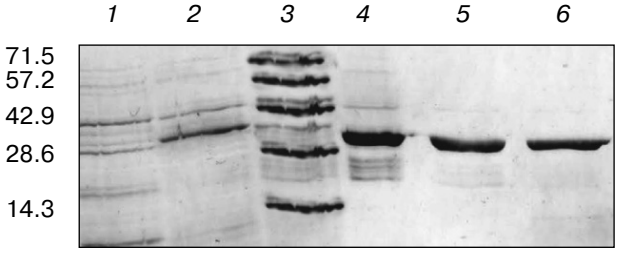

Fig. 3. Electrophoretic separation in 12% polyacrylamide gel in the presence of SDS. Lanes: *1*) cell suspension containing pET-15b-IN (HIV-1)BruB1 before induction (20-times dilution); *2*) the same after induction (20-times dilution); *3*) Dalton Mark III protein markers from Fluka (USA); their molecular masses in kD are given in the left; *4-6*) fractions of HIV-1 (isolate Bru) integrase eluted from Ni-NTA agarose with 100-500 mM imidazole. The gel was stained with Coomassie R-250.

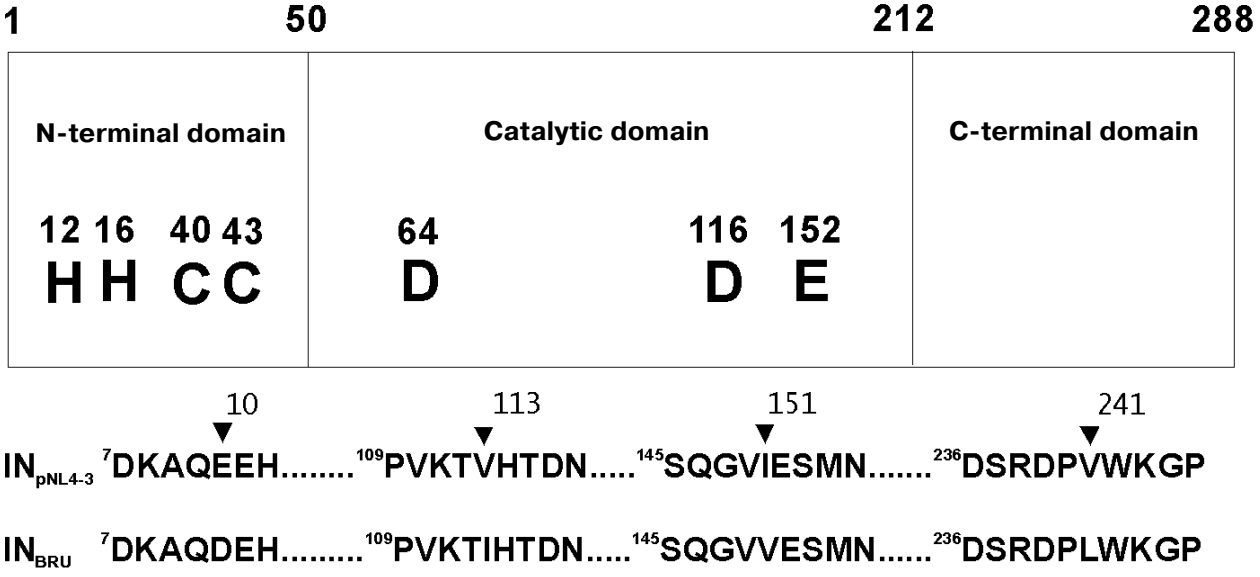

Fig. 4. Scheme of domain structure and distinction of the amino acid sequence of pNL4-3 and HIV-1 (Bru) integrases. Replacement positions are shown by arrows above the amino acid sequence.

due to nonspecific interactions of metal cations and enzyme and to protein precipitation [26].

The ratio of the maximal integrase activity in the presence of Mn^{2+} and Mg^{2+} and the general view of the plot of enzyme activity versus bivalent ion concentration are comparable with earlier data [24, 26]. However, the difference in Mn^{2+} and Mg^{2+} optimal concentrations for the maximal activity of HIV-1 (Bru) integrase is significant: 20 and 40% less than for pNL4-3 integrase, respectively. Using site-directed mutagenesis and limited proteolysis with pLN4-3 integrase, it was shown that independent binding to metal cations occurs in three enzyme sites. The HHCC-motif in the N-terminal domain can independently bind to metal cations via Lys14, in the catalytic domain the binding site is in the sequence of amino acid residues 136-173, and the third site is in the C-terminal domain in the sequence of amino acid residues 258-273 [27].

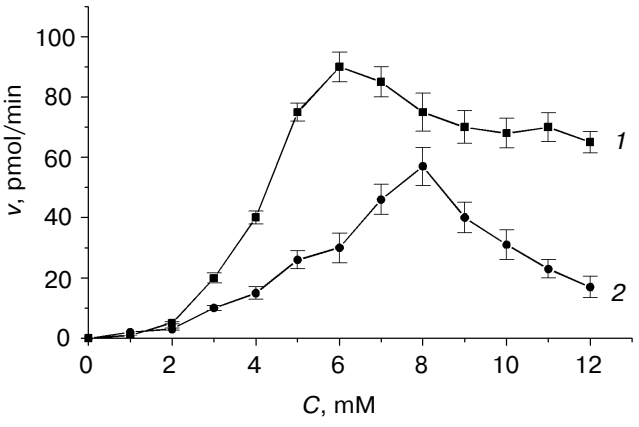

Fig. 5. Rate of formation of products of 3′-processing reaction catalyzed by HIV-1 integrase versus concentration of bivalent cations: *1*) Mg^{2+}; *2*) Mn^{2+}.

Thus, change in the enzyme conformation by the action of Mn^{2+} and Mg^{2+} and consequently, integrase activity significantly depend on the tertiary structure in the binding sites of bivalent metal cations. This can probably account for the difference in Mn^{2+} and Mg^{2+} optimal concentrations for the studied enzyme and pNL4-3 integrase.

To study the interaction of HIV-1 recombinant integrase with earlier described enzyme inhibitors, we chose aurintricarbonic acid which efficiently suppresses HIV-1 replication in infected cell culture and inhibits HIV-1 integrase [28].

The data on electrophoresis of accumulated products of the 3′-processing reaction versus concentration of aurintricarbonic acid are presented in Fig. 6a. The results of several parallel experiments on inhibition of HIV-1 integrase by aurintricarbonic acid are presented in Fig. 6b. At concentration of this acid 6.3 ± 0.4 μM, the relative enzyme activity decreases by 50% ($[I]_{50}$); this corresponds to data on inhibition of recombinant integrase based on pNL4-3 (5 μM) [28].

Consequently, the difference in amino acid sequence of the studied enzyme and pLN4-3 integrase does not effect inhibition by aurintricarbonic acid.

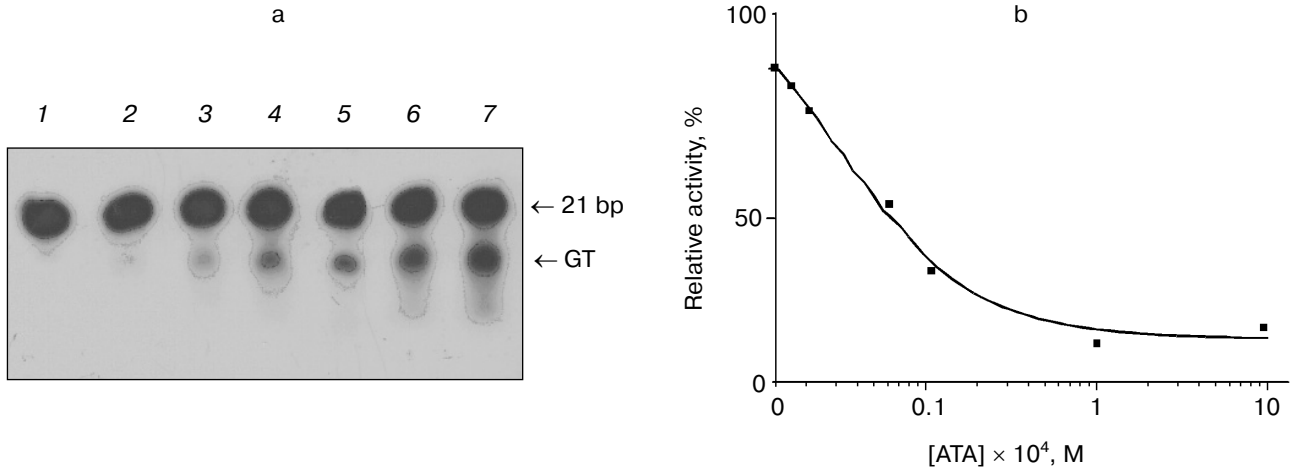

Fig. 6. Inhibition of the 3′-processing reaction catalyzed by HIV-1 integrase by aurintricarbonic acid (ATA). a) Electrophoretic isolation of the reaction products (20% polyacrylamide gel, 7 M urea). ATA concentration (μM): 100 (*2*), 10 (*3*), 1 (*4*), 0.5 (*5*), 0.1 (*6*), 0 (*7*); without integrase (*1*). Positions of oligonucleotide duplex (21 bp) and processed dinucleotide (GT) are given at the right. b) Relative activity of integrase in the 3′-processing reaction versus ATA concentration.

So, we produced functionally active HIV-1 (isolate Bru) recombinant integrase which allows comparison of testing results of HIV integrase inhibitors on *in vitro* isolated enzyme and in cell culture infected by HIV isolate encoding the same version of recombinant integrase.

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