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Selection of *E. coli* Strains for Stable Transformation with Recombinant Plasmids Containing Full-Length Genome of Clinical HIV-1 Isolates

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Strain χ6007 obtained from the parent *E. coli* strain χ5097 is a result of ptsH5 mutation, which allowed cells to grow without common components of the phosphoenolpyruvate-dependent phosphotransferase system. Segregants of strain χ6007 retaining the Pol^+ gene responsible for inability to grow at 37°C, but gaining rifampicin resistance (Rif^R) were used for cloning of cointegrate plasmids. Preintegration complexes of HIV-1 were cointergated with the pBR-322 plasmid and transformed strain χ6018. Sequencing showed that the pPIC91 hybrid plasmid contains full-length genome of HIV-1 with shortened 5-terminal LTR and full-length copy of pBR322. Elimination of the pPIC91 plasmid from χ6018 cells was followed by the appearance of auxotrophic insertion mutants. Sequencing of the insert region showed that chromosome DNA of the host cell includes integrated genomes of pBR-322 and HIV-1.

Key Words: *retroviruses; type I human immunodeficiency virus; HIV-1 cointegration; HIV-1 cloning*

Wide distribution of type I human immunodeficiency virus (HIV-1) in Russia [8] necessitates introduction of biomolecular methods into the diagnostic practice. The first stage consists in cloning of HIV-1 with clinical samples.

The technique of cointegration allows us to obtain complete clones of viral genome not distorted by amplification. Published data show that hybrid plasmids containing preintegration complexes (PIC) of HIV-1 are unstably inherited in *E. coli* K12 cells [14].

This work was designed to select host strains of *E. coli* for cloning of HIV-1 PIC in the pBR322 plasmid.

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MATERIALS AND METHODS

Bacterial strains and plasmids are described in Table 1.

Bacterial cultures of *E. coli* were grown on a liquid or agarized LA medium (1.5% agar). Antibiotics were added to selective media in a concentration of 50 μg/ml.

PIC were obtained and used for cloning. Human peripheral blood mononuclear cells were isolated by centrifugation on a Ficoll-400 step gradient [5], activated with phytohemagglutinin (titer 2×10^7), added to sera (kept at -70°C before assay) containing 1.0-2.5 μg/ml viral p24 protein (~10^7 viral particles per 1 ml), and incubated for 6-8 h [6,7]. The cells were transferred to buffer K containing 20 mM HEPES (pH 7.3), 150 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 U/ml aprotinin, and 0.05% triton X-100. PIC were

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TABLE 1. Cell Strains and Plasmids

Name		Sex, properties	Genotype	Source
Cells	χ5097	F^-	Thi-1, D (lac — proAB)$_{13}$, rpsL31	G. B. Smirnov (N. F. Gamaleya Institute of Epidemiology and Microbiology)
	χ6007	F^-	χ5097, ptsH5	Present work
	χ6008	F^-	χ5097, Rif^R, ptsH5	Present work
	χ6018	F^-	χ5097, Rif^R	Present work
	HB101	F^-	http://molbiol.edu.ru	[11]
	XL1	F^-	http://molbiol.edu.ru	[11]
	DH5α	F^-	http://molbiol.edu.ru	[11]
Plasmids	pBR322	Tc^R, Ap^R	K00005 (GenBank)	[9]
	pPIC91	HIV1, Ap^R	—	Present work
	pNL4-3	HIV1, Ap^R	AF324493 (GenBank)	[4]

pelleted by centrifugation at 8000 rpm for 15 min. DNA content in samples was measured spectrophotometrically. DNA from samples (25 ng) and pBR322 plasmid (25 ng, 100 μl) was transferred to buffer A containing 10 mM $MgCl_2$, 20 mM HEPES (pH 7.2), 10% PEG8000, 5 mM aprotinin, and 1 mM dithiothreitol and maintained in a thermostat at 25°C for 30 min, transformation of *E. coli* competent cells [11] and transduction with bacteriophage Plvir were performed [2].

The polymerase chain reaction (PCR) were carried out using Taq- or Pfu-polymerases. Sequences of primers are shown in Table 2.

The sequence of amplified fragments was estimated on a 373A Automatic Sequencer (Applied Biosystems).

The following software was used: primer counting (PrimPCR [v. 1.0]; Oligo [v. 5.0]; Blast site), sequence bank (Entrez site), sequence analysis (GeneRunner [v. 3]; Dnastar [v. 5]), gel-documentation system (DNAimeger [Promega, v. 2.0]), gel analysis (DNA-analysis [Promega, v. 2.5]), and mathematical and statistical treatment (StudyWKS [v. 4.5]).

RESULTS

For choosing cloning conditions, the cells from 5 strains of *E. coli* K12 were transformed with the pNL4-3 plasmid containing full-length HIV-1 genome (No. AF324493 GenBank) and ampicillin resistance gene (Amp^R, Table 3).

pNL4-3 plasmid-carrying cells produced the Amp^R offspring only on 1.5% agarized medium LA with ampicillin. It should be emphasized that the offspring of cells transformed with the pBR322 plasmid was also obtained on McConkey medium. These results attest to increased permeability of the cell wall in Amp^R cells treated with the pNL4-3 plasmid. The sensitivity of pNL4-3 transformants to oxygen [1] can be effectively compensated by treating the cells with undecyl viologen, an inhibitor of free-radical chain branching reaction. Administration of 100 pM undecyl

TABLE 2. Structure of Amplification Primers

Name and region	Structure of primer (5'→3')	Annealing temperature in PCR, °C
pol-for	CACCTGGATTCCTGAGTG	56
pol-rev	CCCAATGCATATTGTGAG	52
LTR-for	AATTACTCCCAAAGAAGAC	56
LTR-rev	AAAGGGTCTGAGGGATCTC	58
vpr-for	ATGGAACAA(A/G)CCCCAG(A/C)A G(A/C)	58
vpr-rev	(C/T)(C/T)AGGATCTACTGG(A/C)TCCAT	58
ExLTR-1	(^{32}P-)GTCTTCTTTGGGAGTAATT	52
ExLTR-2	(^{32}P-)TGCTGGCTCATAGGGTGTA	58
pBR322-diffused	(N_{15})TTTCT	
E. coli-diffused	(N_{14})TGAAAA	

TABLE 3. Frequency of Cell Transformation with Plasmids and Elimination (lg N/μg)

Parameter	HB101	XL1	χ5097	χ6007	DH5α
pBR322 (4362 bp)	8/nd	8.5/-8	7.5/-8	8/-8	8/nd
pNL4-3 (14824 bp)	2/nd	1.5/nd	1.2/-6	4.5/-6	1.5/nd
pNL4-3(*)	7.5	—	—	7.5/-6	—

Note. Results of the standard experiment. Selective medium LA (1.5%) with ampicillin. nd, not determined. *Inoculation of transformants into undecyl viologen-containing media.

viologen into the selective medium considerably increased the number of Amp^R transformants carrying the pNL4-3 plasmid (Table 3). Further experiments showed that strain χ6007 obtained from the parent strain of *E. coli* χ5097 and carrying the ptsH5 mutation allowing cells to grow without common components of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) [3] best suited for our purposes. Transformants of strain χ6007 grew only at 30°C and were characterized by high instability of the pNL4-3 plasmid.

Further analysis revealed segregants capable of growing at 37°C among Amp^R transformants of strain χ6007 (0.001%). These segregants retained Pol^+ gene responsible for the inability to grow at 37°C [9], but gained rifampicin resistance (Rif^R). This antibiotic interacts with the β'-subunit of *E. coli* RNA polymerase [12]. The segregant inheriting plasmid pNL4-3 and retaining *pol* and *vpr* genes and LTR regions received the name χ6008.

Thus, χ6008 cells passed in undecyl viologen-containing media without common components of PTS stably inherited the pNL4-3 plasmid carrying full-length HIV-1 genome. Strain χ6018 was obtained by the transfer of locus Rif^R into the parent strain χ6007 (Table 3). Strains χ6008 and χ6018 were used to clone cointegrate plasmids.

Samples for cloning of PIC were prepared from 5 HIV-positive sera with high titer of protein p24. Strain χ6018 was used for transformation. Sequences of the HIV-1 provirus were estimated by blot hybridization [11]. In 16 experiments the percentage of clones carrying hybrid plasmids of the specified size was not less than 15%. PCR confirmed the presence of *pol* and *vpr* genes and LTR regions in DNA samples obtained from 22 randomly selected Amp^R clones and exposed to 10-fold passage on selective media. One of the cloned hybrid plasmids (pPIC91) was subjected to 8-fold passage and used for nucleotide sequencing. pPIC91 contained full-length genome of HIV-1 with shortened 5-terminal LTR (Fig. 1, *a*) and full-length copy of pBR322. Thus, inheritance of HIV-1 PIC-carrying plasmids by χ6018 cells was not accompanied by structural changes in PIC. The nucleotide sequence of HIV-1 inserts in pPIC91 was similar to the AF414006 sequence in a blood isolate of HIV from a Belarussian citizen (98% homology) [10].

Previous studies showed that integration of transposon IS481 is stimulated in the presence of common PTS components (*e.g.*, fructose) in the medium. This scheme was used to study functional activity of HIV-1 integrase, whose gene serves as a constituent of the pPIC91 plasmid. Published data show that cloned integrase of HIV-1 had functional activity in *E. coli* cells [13].

Elimination of the pPIC91 plasmid from χ6018 cells grown in the presence of 1% fructose was followed by retention of the Amp^R marker (frequency 10^{-7} per cell), which is typical of the IS481 transfer. According to the nomenclature of insertion mutations AceK::pPIC91, several Amp^R clones not having extrachromosomal DNA act as auxotrophic insertion mutants (for example, by serine). The frequency of Ser→ Ser^+ reversion is below 10^{-7}. Most Ser^+ clones have the Amp^S phenotype. The frequency of Ser^-Amp^R→ Ser^+Amp^R reversion is less than 10^{-8}. Ser^+Amp^R clones often gain secondary auxotrophic activity. Extrachromosomal DNA is absent in the offspring of secondary auxotrophs and parent Ser clone. It can be assumed that auxotrophic mutants are characterized by integration of the plasmid pPIC91 sequence, which is followed by the plasmid transfer to new sites and induction of new auxotrophs.

We studied the nucleotide sequence in site AceK::pPIC91 for the assumed integration into host cell chromosomes. AceK::pPIC91-containing DNA was transferred from strain χ6018 to strain χ5097 by the method of phage transduction. This procedure allowed us to avoid artifacts in sequencing. Fig. 1 shows the results of sequencing of amplification products with pairs of primers ExLTR-1—*E. coli* and ExLTR-2—*E.coli*. A comparative study was performed with the nucleotide sequence of HIV-1, pBR-322, and entire genome of *E. coli* (No. U00096). The cointegrate plasmid of strain χ6018 contained the entire genome of pBR-322 and integrated genome of HIV-1 (Fig. 1, *a*). Integration into the plasmid occurred between nucleotides 1812 and 1813 of pBR-322 and was accompanied by duplication of a 7-membered ID-sequence in the CAGTAAC plasmid (1806-1812). The proviral fragment of HIV-1 in this cointegrate included com-

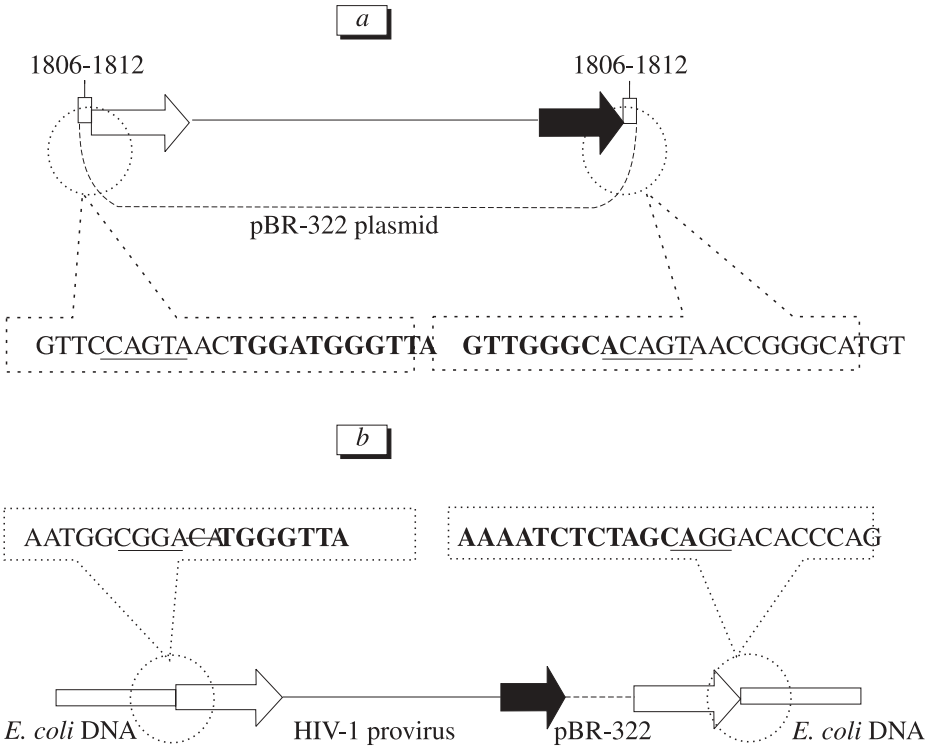

Fig. 1. Products of HIV-1 genome integration. *a*) Scheme of the cointegrate pPIC91 plasmid. Numbers of nucleotides in the pBR-322 plasmid map (K00005, GenBank) designate regions of a duplicate ID-sequence. Nucleotide sequences of junctions between HIV-1 LTR (bold type) and pBR-322. Underlining: ID-sequence. *b*) Scheme for integration of the pPIC91 plasmid into *E. coli* chromosomal DNA. Nucleotide sequences of junctions between HIV-1 LTR (bold type) and *E. coli* DNA. Underlining: chromosomal ID-sequence. Light arrow: full-length HIV-1 LTR (645 bp). Dark arrow: defective HIV-1 LTR (415 bp).

plete 5'-terminal LTR (U3-R-U5 regions, 645 bp), entire viral genome (between LTR), and defective 3'-LTR lacking the 3-terminal region (415 bp, Fig. 1, *a*).

The pPIC91 plasmid was integrated between nucleotides 4217288 and 4217289 of the *E. coli* genome. Integration resulted in duplication of a 5-membered ID-sequence of the bacterial GGACA chromosome (4217284-4217288) adjacent to LTR (Fig. 1, *b*). During integration the complete copy of 3'-terminal LTR was probably recovered by the sequence of 5'-LTR in the pPIC91 plasmid. Four terminal nucleotides of left LTR were lost after integration (Fig. 1, *b*).

It can be hypothesized that integration of pPIC91 involves HIV-1 integrase. This assumption is confirmed by the recovery of 3'-terminal LTR and duplication of a 5-nucleotide ID-sequence typical of HIV-1. *In vitro* cointegration of HIV-1 PIC and pBR-322 leads to duplication of a 7-membered ID-sequence. This process probably differs from *in vivo* integration.

The proposed laboratory method allowed us to clone HIV-1 PIC in the pBR322 plasmid (up to 10^8 clones/μg DNA) and obtain stable offspring in *E. coli* cells. Our results indicate that *E. coli* carries the gene of HIV-1 integrase, which is responsible for PIC DNA insertion into the chromosome of host cells.

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REFERENCES

1. G. I. Karataev, L. M. Sinyashina, and I. G. Sivov, *Vestn. Ros. Akad. Med. Nauk*, No. 1, 34-38 (2000).
2. I. G. Sivov, O. A. Belyavskii, and G. I. Karataev, *Mol. Genet*., No. 2, 33-36 (2000).
3. I. G. Sivov, T. N. Bol'shakova, and G. I. Karataev, *Genetika*, **37**, No. 7, 900-907 (2001).
4. A. Adachi, H. E. Gendelman, S. Koenig, *et al*., *J. Virol*., **59**, No. 2, 284-291 (1986).
5. A. C. Berardi, A. Wang, J. D. Levine, *et al*., *Science*, **267**, 104-108 (1995).
6. V. Ellison, H. Abrams, T. Roe, *et al*., *J. Virol*., **64**, No. 9, 2711-2715 (1990).
7. C. M. Farnet and W. A. Haseltine, *Proc. Natl. Acad. Sci. USA*, **87**, No. 9, 4164-4168 (1990).
8. S. C. Kalichman, J. A. Kelly, K. J. Sikkema, *et al*., *Int. J. STD AIDS*, **11**, No. 2, 71-76 (2000).
9. B. Kim and L. A. Loeb, *Proc. Natl. Acad. Sci. USA*, **92**, No. 1, 684-688 (1995).
10. K. Liitsola, K. Holm, A. Bobkov, *et al*., *AIDS Res. Hum. Retroviruses*, **16**, No. 11, 1047-1053 (2000).
11. J. E. Sambrook, E. F. Fritsch, and T. Maniatis, *Molecular Cloning: A Laboratory Manual*, New York (1989).
12. K. M. Severinov, A. Soushko, A. Goldfarb, and V. Nikiforov, *Mol. Gen. Genet*., **244**, No. 1, 120-126 (1994).
13. M. Sioud and K. Drlica, *Proc. Natl. Acad. Sci. USA*, **88**, No. 11, 7303-7307 (1991).
14. P. Zervos, T. Hassell, R. Van Frank, and M. T. Lai, *Biochem. Biophys. Res. Commun.*, **170**, No. 5, 1061-1066 (1990).