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The Titer of the Lentiviral Vector Encoding Chimeric *TRIM5α-HRH* Gene is Reduced Due to Expression of TRIM5α-HRH in Producer Cells and the Negative Effect of Ef1α Promoter

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The chimeric protein TRIM5α-HRH is a promising antiviral factor for HIV-1 gene therapy. This protein is able to protect cells from HIV-1 by blocking the virus in the cytoplasm. We are developing protocol of HIV-1 gene therapy, which involves the delivery of the *TRIM5α-HRH* gene into $CD4^+$ T-lymphocytes by lentiviral vectors (LVs). However, LVs containing *TRIM5α-HRH* have a low infectious titer, which prevents effective T cell modification. Here, we found that the expression of *TRIM5α-HRH* during pseudoviral particle production in HEK293 T cells, as well as the presence of the Ef1α promoter in our construction are responsible for titer reduction. These results allow us to determine the directions for further optimization of LV with the *TRIM5α-HRH* gene to improve its infectious titer.

Keywords: TRIM5α, TRIM5α-HRH, gene therapy, HIV-1, Ef1α, lentiviral vector

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INTRODUCTION

HIV infection is currently an incurable disease. Antiretroviral (ARV) drugs suppress viral replication and reduce viral load, but do not completely eliminate the virus from the body. For this reason, HIV-infected patients are forced to take ARVs for life, which is almost always associated with side effects. The regimen of taking these drugs must be strictly observed, otherwise the risk of emergence of ARV-resistant variants of the virus increases [1, 2]. These problems force us to look for new approaches to the treatment of HIV-1, one of which is gene therapy, which involves the introduction of virus-blocking genes into the $CD4^+$ T lymphocytes or $CD34^+$ stem hematopoietic progenitors, which give rise to all cell populations involved in the development of infection [3, 4]. Lentiviral vectors (LVs) constructed on the basis of HIV are currently considered the most effective delivery means. They are able to efficiently deliver the transgene to $CD4^+$ T-lymphocytes, ensure its integration into the genome, maintain stable expression and the possibility of transmission to daughter cells during proliferation [5]. The safety of LVs has been confirmed by numerous preclinical and clinical studies of drugs based on these vectors for the treatment of various pathologies [6–14].

TRIM5α proteins (TRIpartite Motif, 5α-isoform) are capable of blocking various retroviruses in the cytoplasm, and the spectrum of neutralized viruses is specific for different mammalian species [15, 16]. Thus, the TRIM5α protein of rhesus monkeys TRIM5α-rh (TRIM5α rhesus) is able to prevent HIV-1 infection, while the human homologue huTRIM5α does not protect cells from HIV-1 [17]. The chimeric protein TRIM5α-HRH (human-rhesus hybrid) created in 2005 is a modified huTRIM5α, in which 11 amino acid residues in the PRYSPRY domain are replaced by 13 amino acid residues from TRIM5α-rh [18]. As a result of this change, the TRIM5α-HRH protein acquired the ability to inhibit HIV-1 replication in human cells [19].

TRIM5α-HRH is a promising factor for GT of HIV-1 infection. However, there are difficulties with the production of LVs containing this gene. During production such LVs have a low titer [19, 20], which prevents the effective modification of $CD4^+$ lymphocytes and complicates the further development of GT. This work is devoted to the search for the reasons for the low titer of LVs containing *TRIM5α-HRH*.

Table 1. *TRIM5α* gene reamplification primers, matrices and names of the resulting constructs

Primer (5'→3')	Matrix	Construct
agcgaattccgccaccatggcttctggaatcctgg, gttctagattatcaagagcttggtgagcacagagt	pGEM-TRIM-HRH	TRIM-HRH-LV
agcgaattccgccaccatggcttctggaatcctgg, gttctagattatcaagagcttggtgagcacagagt	pGEM-huTRIM	huTRIM-LV
agcgaattc**taa**gcttctggaatcctggttaatgta, gttctagattatcaagagcttggtgagcacagagt*	pGEM-TRIM-HRH47TAA	^{stop}TRIM-HRH-LV
agcgaattc**taa**gcttctggaatcctggttaatgta, gttctagattatcaagagcttggtgagcacagagt*	pGEM-huTRIM47TAA	^{stop}huTRIM-LV
agcgaattc**taa**gcttctggaatcctggttaatgta, gttctagattaccatccacaccctgaagcagctcca*	pGEM-huTRIM	^{stop}½huTRIM-N-LV
agcgaattcgcttcagggtgtggatggcgtcat, gttctagattatcaagagcttggtgagcacagagt	pGEM-TRIM-HRH	½TRIM-HRH-C-LV
agcgaattcgcttcagggtgtggatggcgtcat, gttctagattatcaagagcttggtgagcacagagt	pGEM-huTRIM	½huTRIM-C-LV
gaactcgagcgccaccatggcttctggaatcctgg, gttctagattatcaagagcttggtgagcacagagt	pGEM-TRIM-HRH	CMV-TRIM-HRH-LV

* Reamplification with this pair of primers led to the replacement of the atg codon at position 1 by **taa**.

EXPERIMENTAL

Cloning of constructs. The LVs were constructed on the basis of the pLVX Puro vector (Clontech, USA) containing intact long terminal repeats (LTR), a CMV promoter, and a PGK-puro expression cassette. The cPPT element (central polypurine tract) was replaced with the longer sequence of cPPT using the previously described technique, and the selective marker of puromycin resistance *puro* was replaced by the green fluorescent protein (eGFP) gene [21]. The resulting construct was named pLVX eGFP. The eGFP-LV construct was obtained by deleting the CMV promoter sequence from pLVX eGFP at the ClaI and BamH1 sites. To obtain Ef-PGK-eGFP-LV, the CMV promoter in pLVX eGFP was replaced with Ef1α at the ClaI and BamH1 sites. The Ef1α sequence was obtained by amplification of human genomic DNA with the primers atcgataccgtcagtgggcaga and ggatccaggggtagttttcacga. Then, a linker with the necessary restriction sites obtained by annealing the oligonucleotides gatcctgctagaattctcagcatatggtct and ctagagaccatatgctgagaattctagcag was inserted into Ef-PGK-eGFP-LV at the BamH1 and XbaI sites. The genes *huTRIM5α* and *TRIM5α-HRH* were obtained according to [21] and cloned into pGEM-T (Promega, United States). In the plasmids pGEM-huTRIM and pGEM-TRIM-HRH, the atg codon at position 47 was changed by site-directed mutagenesis to taa. The resulting constructs was named pGEM-huTRIM47TAA and pGEM-TRIM-HRH47TAA. Further *TRIM5α* sequences were re-amplified and inserted at EcoR1 and XbaI sites in Ef-PGK-eGFP-LV. To obtain CMV-TRIM-HRH-LV the amplified gene *TRIM5α-HRH* was embedded in pLVX eGFP at the XhoI and XbaI sites. Primers for reamplification, templates, and the resulting constructs are shown in Table 1.

To obtain the noEf-TRIM-HRH-LV construct, the Ef1α promoter was removed from the TRIM-HRH-LV vector at the ClaI and BamH1 sites, and the vector was ligated to itself.

Cell lines. SupT1 lymphoblastoid cells (ATCC CRL-1942) were cultured in Advanced RPMI1640 medium (Thermo Fisher Scientific, United States) supplemented with 2% fetal bovine serum (FBS, Gibco, United States) and L-glutamine up to 4 mM (PanEco, Russia). HEK293T cells (ATCC® CRL-3216) were cultured in DMEM medium (PanEco) with 10% FBS (Gibco) and 4 mM L-glutamine (PanEco). All cell lines were cultured at 37°C and 5% CO_2.

Obtaining lentiviral particles. To obtain particles pseudotyped with VSV-G (protein G of vesicular stomatitis virus), HEK293T cells were transfected with a mixture of second-generation packaging plasmids (0.6 μg pCMV-dR8.91, 0.1 μg pCMV-VSV-G [20]) and the corresponding LV construct (0.6 μg) in 6-well culture plates (TPP, Switzerland)). We used linear polyethyleneimine (PEI MAX 40000; Polysciences Inc., United States). Transfection, collection, and storage of LV particles were carried out according to [20].

Western blotting. Cells of the HEK293T line, collected after the production of lentiviral particles from one well, were lysed in 0.5 mL of Proteojet lysis reagent (Thermo Fisher Scientific). The lysate (10 μL) was used for loading onto a polyacrylamide gel followed by transfer to an Immun-Blot® PVDF mem-

brane (BioRad, United States). The antibodies used were: anti β-actin (ab8227, Abcam, United Kingdom) 1 : 8000, anti TRIM5α (ab59000, Abcam) 1 : 500, secondary anti-rabbit antibodies labeled with horseradish peroxidase (ab6721, Abcam) 1 : 12000.

Determination of the infectious titer of pseudoviral particles. The infectious titer of pseudoviral particles (transduction units/mL, or TU/mL) was determined by titration of the LV suspension on the SupT1 cell line. For this, a series of five-fold dilutions (1, 1/5, 1/25, 1/125) prepared from freshly thawed LV aliquots was used for transduction of 10^5 cells in the presence of 2 μg/mL polybrene in a volume of 0.3 mL on a 48-well plate (TPP, Switzerland). The next day, the growth medium was replaced with a fresh one. 48 h after transduction, the proportion of cells expressing the eGFP marker ($eGFP^+$ cells) was measured using a NovoCyte Quanteon flow cytometer (Agilent Technologies, USA). The titer was calculated using the formula: $a \times 10^5 \times d/v$, where a—the proportion of $eGFP^+$ cells, 10^5—the total number of cells, d—virus dilution factor, v is the volume of virus added to the well. For the calculation we used dilutions which provide transduction efficiency no more than 30%. To determine the titer by quantitative PCR, SupT1 cells obtained after transduction with LV dilutions were cultured for 3 weeks in order to get rid of the residual plasmid DNA introduced into the culture together with the LV particles. The cells were treated with benzonase (250 kU/mL, Merck, Germany) at a dilution of 1 : 37000 for 1 h at 37°C. DNA was isolated from cell lines transduced with different amounts of the vector and the number of vector copies inserted into the genome was determined using the primers ccttgtataaatcctggttgctgtct, ggaaaggagctgacaggtggt and the R6G-tcaggcaacgtggcgtggtgtg-BHQ2 probe. To normalize vector copy number (VCN) to the number of cells, the β-globin gene was used (primers gtcagggcagagcctctattgct, ccacatgccagtttctattggtct and the Fam-tgcccagggcctcaccacca-BHQ1 probe), obtaining an average VCN per cell. The SupT1 cell line containing 1 copy of the inserted LV per cell was used as a quantitative standard. A graph of the dependence of the average VCN per cell on the volume of added LV (dilutions 1/125, 1/25, and 1/5) was built in MS Excel. According to the equation of the linear approximating curve, the infectious titer of the vector was calculated as the number of transduction units in 1 mL of the viral suspension.

RESULTS

The Proteins TRIM5α-HRH and huTRIM5α Reduce LV Titer

One of the reasons for the decrease in the titer of LVs containing *TRIM5α-HRH* may be the presence of the TRIM5α-HRH protein in HEK293T producing cells, since its homolog—protein TRIM5α-rh—has the ability to reduce the production of HIV-1 and LVs in HEK293T cell cultures [22, 23]. Expression of TRIM5α-HRH during accumulation of the LV is provided by a plasmid encoding a vector RNA.

The effect of TRIM5α proteins on LV titer was assessed using LV constructs shown in Fig. 1a. TRIM-HRH-LV and huTRIM-LV constructs contain the *TRIM5α-HRH* and *huTRIM5α* genes under the control of the Ef1α promoter, as well as the *eGFP* marker under the control of the PGK promoter. Constructions ^{stop}TRIM-HRH-LV and ^{stop}huTRIM-LV lack an ATG start codon and contain stop codons at the beginning of the reading frame, which prevents the initiation of protein translation (see Experimental). As a control LV, we used eGFP-LV containing only a cassette for expression of the marker gene (Fig. 1a). These constructs were used to generate pseudoviral particles (Fig. 1b). We assessed the effectiveness of translation suppression by determining the amount of TRIM5α protein in HEK293T-producing cells using Western blotting (Fig. 1b). A significant amount of protein was found in cells with the TRIM-HRH-LV vector. In cells with the vector ^{stop}TRIM-HRH-LV there was a weak signal, the same as in cells with eGFP-LV. The signals in ^{stop}TRIM-HRH-LV and eGFP-LV may be appear due to the presence of the endogenous protein TRIM5α.

The titers of TRIM-HRH-LV and huTRIM-LV were 20 times lower than the titer of the control vector (Fig. 1c). It was found that the suppression of translation of TRIM5α proteins in LV structures ^{stop}TRIM-HRH-LV and ^{stop}huTRIM-LV doubles their production (Fig. 1c). Therefore, both proteins—TRIM5α-HRH and huTRIM5α—reduce the titer of LVs.

When assessing the infectious titer, we drew attention to the fact that the median of the eGFP fluorescence intensity (MFI) in cells with constructs that contain the *TRIM5α* genes under the Ef1α promoter, was approximately 4.5 times lower than in cells with a control vector (Fig. 2a). Decreased fluorescence intensity of the marker makes difficult to separate $eGFP^+$ and $eGFP^-$ populations of cells by a flow cytometry, which can reduce the sensitivity of determining the LV titer. To exclude underestimating the titer when it was measured using a fluorescent marker, we estimated the titer in an alternative way. Genomic DNA was isolated from the cell lines, which were used to measure eGFP fluorescence for titer assessment by quantitative PCR. Measurement of the titer of each of the LVs presented in Fig. 2b using PCR gave values similar to the values obtained by measuring the titer by eGFP fluorescence (Fig. 1c). Thus, in this case, the eGFP marker correctly reflects the infectious titer of the LVs.

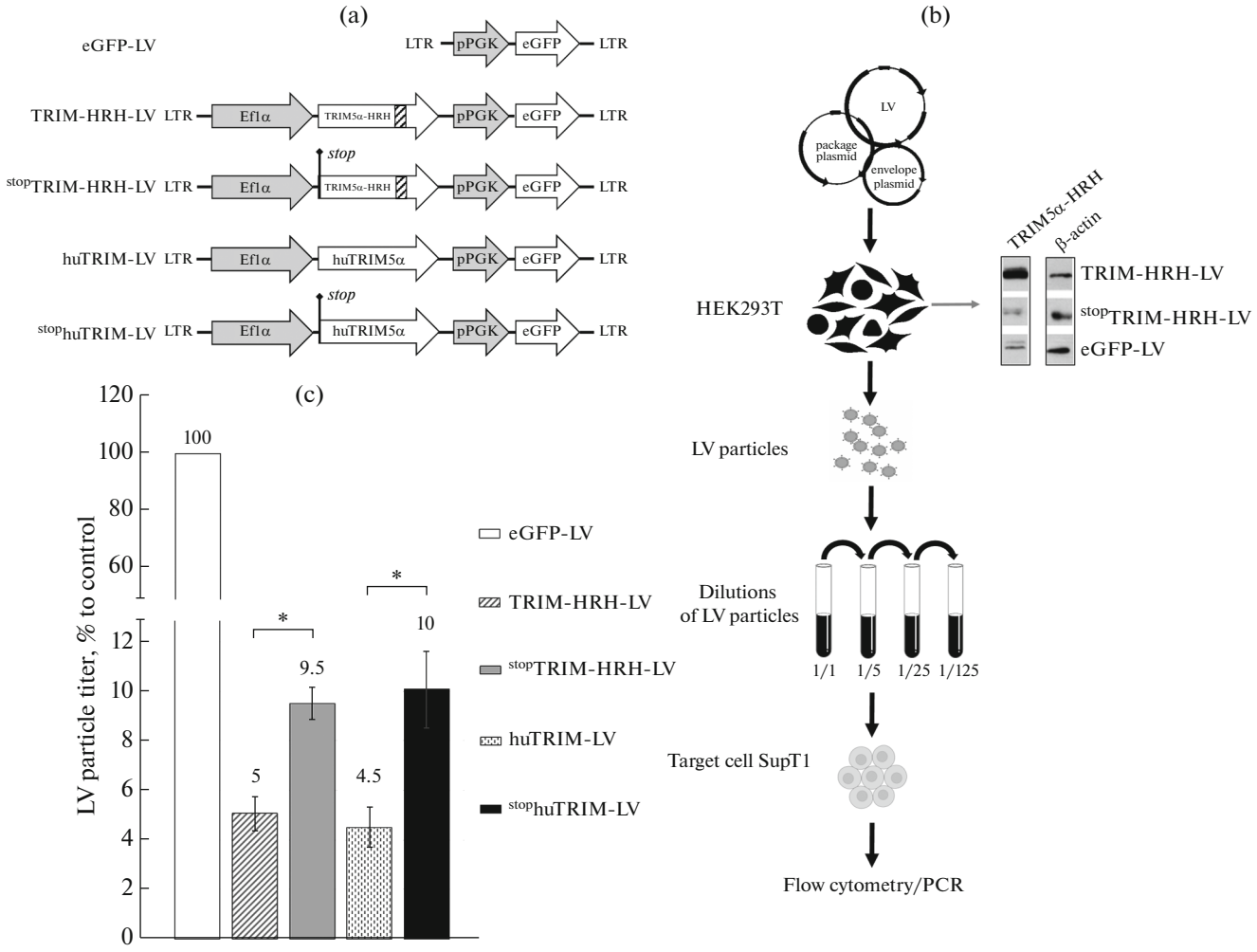

Fig. 1. Assessment of the effect of TRIM5α proteins on LV production. (a) LV-structures schemes: LTR—long terminal repeat, Ef1α—human elongation factor 1 gene promoter, PGK—promoter mouse phosphoglycerate kinase gene; "Stop"—the position of the stop codon; shaded rectangle in the *TRIM5α-HRH* gene frame indicates the sequence coding for the PRYSPRY-domain region containing amino acids from TRIM5α-rh; (b) experimenal design and Western blot analysis of TRIM5α protein in HEK293T cells. When creating the scheme of the experiment, images from the Biorender resource were used. (c) The infectious titers of the constructs determined on SupT1 cells are shown. The infectious titer of the control vector eGFP-LV, taken as 100%, was 1×10^7 TU/mL. *$p > 0.05$ according to the Mann–Whitney test, $n = 4$.

The Nucleotide Sequence of the TRIM5α Gene Per Se does not Affect the Production of Lentiviral Vectors

The revealed effect of TRIM5α proteins on the LV titer (titer reduction by 2 times) does not fully explain the 20-fold difference between the control vector and vectors containing *TRIM5α-HRH* and *huTRIM5α*. We suggested that another reason for the low titer could be the nucleotide sequences of the *TRIM5α* genes per se. To test this assumption, we shattered the *TRIM5α-HRH* and *huTRIM5α* genes into two equal parts and inserted them into the LV under the Ef1α promoter. In this way we produced the construct stop½huTRIM-N-LV containing the N-terminal portion of the gene and two constructs—½TRIM-HRH-C-LV and ½huTRIM-C-LV containing C-terminal regions (Fig. 3a). At the beginning of each half of the *TRIM5α* genes start codons are absent, which excludes translation of truncated protein variants. The two variants of the C-terminal sequence was created due to differences in this part between TRIM5α-HRH and huTRIM5α proteins. We developed the indicated LVs and evaluated their titer. It was expected if one of the half-gene construct contains a sequence unfavorable for LV production, we will see an increase in titer of the construct containing other half. However, the infectious titers of stop½huTRIM-N-LV, ½TRIM-HRH-C-LV and ½huTRIM-C-LV did not differ significantly. The titers of constructs ½TRIM-HRH-C-LV and ½huTRIM-C-LV turned out to be comparable with the titers of LVs with full-length *stop TRIM5α* genes, and the construct stop½huTRIM-N-LV provided an increase in titer only 1.4 times (Fig. 3b). So, there are no structures that significantly affect the LV titer in the *TRIM5α* genes sequences.

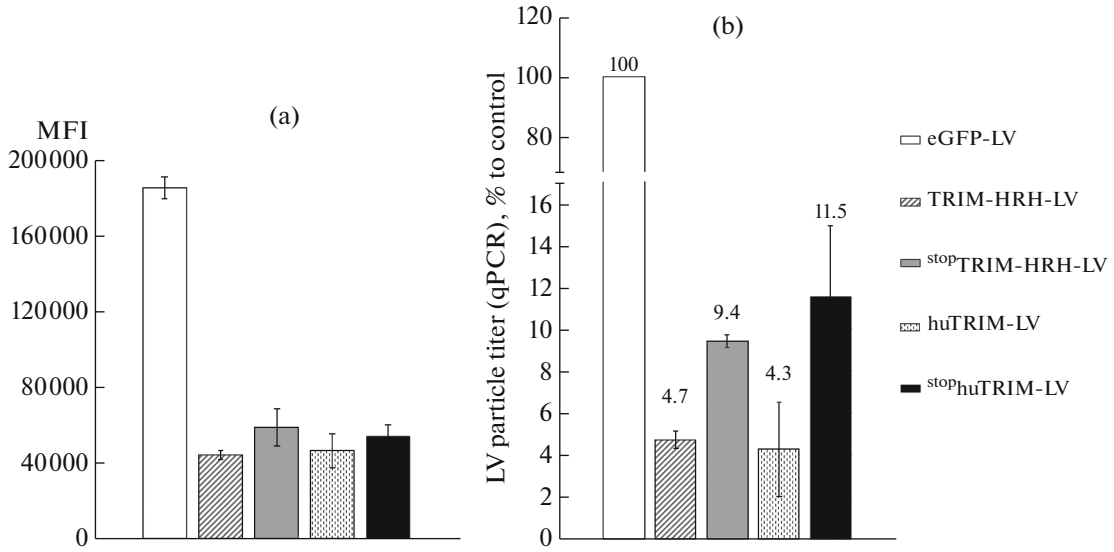

Fig. 2. Median fluorescence intensity and assessment of infectious LV titer using quantitative PCR. (a) Median fluorescence intensity (MFI) in eGFP$^+$-populations of SupT1 cells in determining the infectious titer, $n = 4$; (b) Infectious titer of constructs determined on SupT1 cells using quantitative PCR. The infectious titer of the control vector eGFP-LV, taken as 100%, was 8×10^6 TU/mL; $n = 4$.

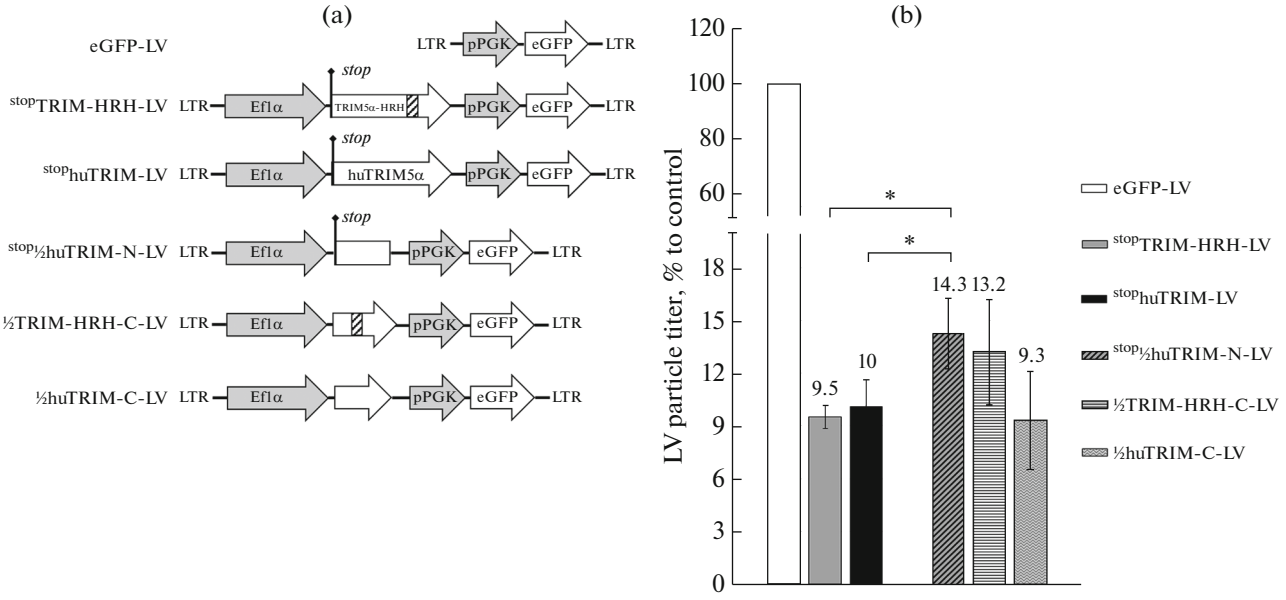

Fig. 3. Analysis of the infectious titer of constructs containing half of the *TRIM5α* genes. (a) Diagrams of LV structures, LV elements are designated as in Fig. 1a. (b) Infectious titer of constructs, determined on SupT1 cells by fluorescence of the eGFP marker. The infectious titer of the control vector eGFP-LV, taken as 100%, was 1×10^7 TU/mL; $^*p > 0.05$ according to the Mann–Whitney test, $n = 4$.

The Ef1α Promoter in the LV Construct Reduces the Titer of Pseudoviral Particles

In addition to the sequence of the target gene and its product, the titer can be influenced by other elements of the LV construct. Analyzing the reasons for the low titer, we focused on the Ef1a promoter, which provided the expression of *TRIM5α-HRH* in our constructs. In order to study of the promoter effect, the following constructs were obtained (Fig. 4a): (1) Ef-PGK-eGFP-LV, in which the Ef1α promoter is retained, but *TRIM5α-HRH* gene sequence is missing; (2) noEf-TRIM-HRH-LV containing only the gene *TRIM5α-HRH*, without the Ef1α promoter; (3) CMV-TRIM-HRH-LV, where we replaced Ef1α with the cytomegalovirus promoter (CMV). All constructs contained the pPGK-eGFP cassette for expression of the marker gene.

The production of LVs and the assessment of their titers allow to establish that the addition of the Ef1α

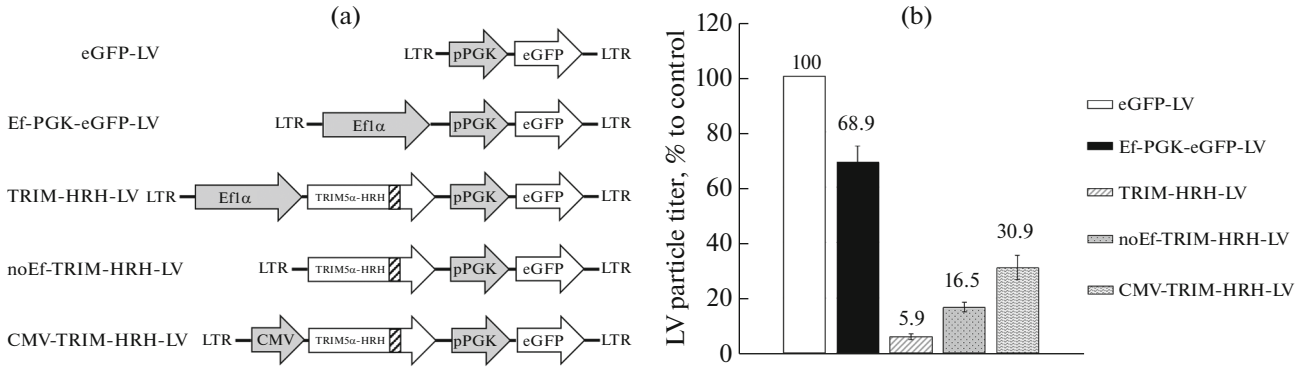

Fig. 4. Effect of the promoter on the titer. (a) Diagrams of LV structures, LV elements are indicated, as in Fig. 1a. (b) Infectious titer of constructs, determined on SupT1 cells by fluorescence of the eGFP marker, $n = 4$. The infectious titer of the control vector eGFP-LV, taken as 100%, was 8×10^6 TU/mL.

promoter to the control vector leads to a decrease in the titer by about 30% (as shown by the analysis of the Ef-PGK-eGFP-LV construct). Removal of Ef1α from the TRIM-HRH-LV construct or its replacement with the CMV promoter leads to an increase in the titer of the noEf-TRIM-HRH-LV and CMV-TRIM-HRH-LV constructs compared to TRIM-HRH-LV by 2.8 and 5.2 times, respectively (Fig. 4b). Therefore, the Ef1α promoter negatively affects the LV titer.

DISCUSSION

Protein TRIM5α-HRH is a promising protective factor for gene therapy of HIV-1, which blocks viral replication [18, 19, 24]. However, LVs encoding the *TRIM5α-HRH* gene have a low infectious titer, which complicates the process of modifying primary $CD4^+$ cells [20] and inevitably increases the cost of production. Determining the causes of the low titer of LVs and finding ways to solve the problem is extremely important, since at the clinical trials this is a serious limitation for the use of this vectors in gene therapy.

We determined the reasons for the low titer of the LVs containing the gene *TRIM5α-HRH* under the control of the Ef1α promoter. During the production of LVs the *TRIM5α-HRH* gene located on a vector plasmid is expressed in the producing cells. We considered the appearance of the TRIM5α-HRH protein during the production of particles as the main reason for the low titer of LVs, since it is known that the TRIM5α proteins are involved in the process of reducing the production of HIV-1 and LVs in HEK293T cells [22, 23]. This phenomenon is called restriction at the late-stage of virus life cycle. In early studies, it was shown that only TRIM5α-rh can provide late restriction [22]. However, later it was found that huTRIM5α also inhibits the production of HIV-1 [25]. In the composition of the chimeric protein TRIM5α-HRH, most of it belongs to the human protein; therefore, one would expect that TRIM5α-HRH can also affect the LV titer. This assumption was confirmed as a result of our experiments—it turned out that the TRIM5α-HRH protein and huTRIM5α equally affect the LV titer, decreasing it by about 2 times. To eliminate this effect, it is necessary to exclude or significantly reduce the expression of TRIM5α-HRH in HEK293T cells during the production of particles. For this purpose, LV production system, which prevents the expression of the gene of interest in producer cells can be used. Potentially promising approaches are described that are applicable to solving the problem [26–28]. However, it should be noted that the effect of the protein was not as significant as expected.

It was found that the presence of the Ef1α promoter lowers the titer of LV particles. The effect of the internal promoter or its position in the vector on the LV titer has been described previously [29, 30]. The most likely reason for this phenomenon is interference between internal promoter and closely located promoters, including the promoter that controls the formation of vector RNA. Thus, Ef1α has the ability to suppress another promoter within LV, which is located upstream of Ef1α [31]. Similarly, the presence of Ef1α in our vector can suppress the LTR promoter, which regulates the production of LV genomic RNA. Competition for binding with transcription factors may underlie the interference between these promoters [32]. Binding sites for the same transcription factors are present in Ef1α and LTR [33, 34].

The interference between Ef1α and the downstream PGK promoter can also explain the decrease in eGFP expression in the constructs shown in Fig. 2a, insofar as this effect was not observed when the Ef1α promoter was removed or replaced with CMV (data not shown). It should be noted that in the future, during the transition to the clinical phase of research, it will be necessary to remove the marker gene and its promoter. Thus, in our case, the interference between internal promoters has no practical significance.

By replacing the Ef1α promoter with the CMV promoter, we managed to significantly (5 times)

increase the titer of the *TRIM5α-HRH* containing vector. However, this construct may be ineffective for gene therapy, since the CMV promoter exhibits weak activity in the target $CD4^+$ cells [35]. In addition, this promoter is susceptible to methylation, and the activity of gene expression under its control gradually decreases [36, 37]. Taking into account the influence of the Ef1α promoter on the titer of LVs carrying the *TRIMα-HRH* gene, it would be advisable to search for an alternative promoter that would not interfere with obtaining a LV titer acceptable for work. In this case, the new promoter should provide long-term expression of the transgene in $CD4^+$ lymphocytes at a level sufficient to protect them from HIV-1, which complicates the task.

We have established at least two reasons for the low titer of LVs containing *TRIM5α-HRH* under the control of the Ef1α promoter,—these are the ability of the TRIM5α-HRH protein to reduce the LV titer during production in HEK293T cells and the presence of the Ef1α promoter in the vector. However, it is possible that other factors also affect the titer. In particular, we considered the sequence of the gene *TRIM5α-HRH* per se as factor, which was not confirmed. In addition, the length of the LV is one of the parameters that affect the titer. It is known that an increase in the size of the LV insert inevitably leads to a decrease in the titer [38, 39]. The Ef1α promoter and the gene *TRIM5α-HRH* consist of 1189 and 1488 bp, respectively, which may affect the LV titer. However, our results indicate that length is not the main reason for the low titer of the TRIM-HRH-LV vector. For example, the length of the CMV-TRIM-HRH-LV vector is 604 bp longer than noEf-TRIM-HRH-LV (Fig. 4a), but the CMV-TRIM-HRH-LV titer is approximately 2 times higher. There can be many reasons affecting the LV titer, and it is not always easy to assess the influence of each factor. It appears that the problem of low titer can be solved using an empirical approach, which, in the future, will make it possible to find the optimal combinations of the elements of LV design.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflicts of interest.

Statement on the welfare of humans or animals. This article does not contain any studies involving humans or animals performed by any of the authors.

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