UDC 578.222

DNA-LAUNCHED INFECTIOUS CLONE WITH AN ARTIFICIAL INTRON: INFLUENCE ON VIRUS RESCUE AND GROWTH

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ABSTRACT

A DNA-launched infectious clone is a plasmid that contains the full-genome cDNA copy of a viral genome under control of a eukaryotic promoter. Transfection of the plasmid in cell culture can then rescue the virus and allow for its growth. In this study, DNA-launched infectious clones of Venezuelan equine encephalitis virus (VEE) were produced. In the developed constructs, a cDNA copy of the VEE genome was placed under control of the cytomegalovirus (CMV) promoter, and a ribozyme and polyadenylation signal were engineered at the′3 - end of the sequence. Moreover, a small hybrid intron (composed from parts of the second beta-globin intron and the IgG intron) was cloned into the junction between the viral′5 -untranslated region (UTR) and the nsP2 gene, and the effects of inserting an intron in the DNA-launched infectious clone on the rescue efficiency and growth kinetics were assessed.The rescue efficiency was high for all constructs at 4.8 × 104 focus-forming units (FFU)/ μg of transfected plasmid DNA for the parental construct, 4.0 × 104 FFU/μg for the construct with a non-natural PstI site between the′5 UTR and nsP2 gene, and 1.0 × 104 FFU/μg for the construct with the intron placed in a selected genomic position. The three rescued viruses reached similar titers, indicating that the intron does not have a major effect on the rescuing efficiency. Thus, we have demonstrated an efficient method of cloning introns into natural or engineered PstI sites to achieve efficient viral rescue and growth.

Keywords: alphavirus, Venezuelan equine encephalitis virus, DNA-launched infectious clone, intron, virus rescue, CMV promoter.

INTRODUCTION

Alphaviruses are RNA-containing viruses, members in the genus Alphavirus, family Togaviridae. Several alphavirus species including Venezuelan equine encephalitis virus (VEE), Sindbis virus and Semliki Forest virus have been utilized as vectors for high-level expression of recombinant proteins [1]. Ongoing studies employ alphaviruses as vectors in live vaccines, gene therapy [2, 3], oncolytic virotherapy and cell engineering [4].

Alphaviruses are attractive as vectors because they have a wide host range and infect multiple cell types, grow to high titers and their genomes are relatively easy to manipulate [5]. The alphavirus genome is positive-polarity capped RNA. Two large open reading frames (ORF) are present in genomic RNA, of which the first ORF occupies 5≥-proximal two thirds of the genomic RNA and encodes 4 non-structural (NS) proteins (nsP1-4). The second ORF occupies the 3≥-proximal 1/3 part of the genomic RNA and encodes viral structural proteins. The NS proteins are components of the viral replicative machinery. During the viral life cycle, the replicase converts the genomic RNA in a double-stranded RNA replicative intermediate. Then the negative-polarity RNA strand in the intermediate serves as a template for the generation of multiple copies of newly synthesized viral genomes, and also a different RNA species is synthesized which is named subgenomic RNA (sgRNA). A region at the 3≥-end of the first ORF is recognized by the viral replicase as a start site to begin a synthesis of sgRNA. Accordingly, this region is termed subgenomic pro-

moter (SGp). sgRNA corresponds to the 3≥-proximal 1/3 part of the genomic RNA and is translated to generate viral structural proteins, i.e. the capsid protein (C) and the shell proteins E3, E2, 6K, E1.

From a point of view of genetic engineering, the alphaviral molecular mechanism for the synthesis of sgRNA is convenient for exploitation to generate heterologous expression constructs. The SGp can be duplicated in a recombinant viral genome and a heterologous gene can be placed under the control of an additional copy of the SGp, resulting in the virus that expresses a heterologous gene.

Studies in reverse genetics of alphaviruses become possible upon development of molecular clones. A molecular clone is a DNA molecule (typically a bacterial plasmid) engineered to carry a cDNA copy of a RNA-genome. Molecular clones can be used to rescue live viruses. Often such plasmids are used as templates for in vitro transcription with a use of a recombinant bacteriophage-derived RNA polymerase [6]. In this approach (naked RNA transfection) a live virus is produced in a cell culture or in vivo upon transfecting the synthetic RNA in the cell culture or living tissue. An important alternative to the naked RNA approach is to utilize cellular transcription machinery to generate genomic RNAs. In the majority of such studies, a cDNA-copy of the viral genome is cloned downstream of a strong eukaryotic promoter, e.g. the cytomegalovirus (CMV) immediate-early promoter. To ensure formation of transcripts with correct 5≥- and 3≥-ends, the cDNA-copy is flanked with ribozymes, such as a hammerhead ribozyme upstream of the 5≥-end and the antigenomic ribozyme of hepatitis D virus downstream the 3≥-end. Transcription directed from the CMV promoter occurs in the nucleus, then RNA transcripts are exported to the cytoplasm, where they are being translated and initiate replication. From this point the replication is no more dependent from the nuclear transcription. Plasmids designed to initiate viral replication upon cell entry are termed DNA-launched infectious clones [7].

The approach of DNA-launched infectious clones is considered as a technology for self-amplifying DNA vaccines , although it has advantages not related to the vaccine field. One of such advantages is that the approach allows generating RNA genomes of practically unlimited sizes, whereas the best known methods of *in vitro* transcription restrict the maximal size of RNA transcripts to ~15 kb. For example, the DNA-launched infectious clone approach allowed rescuing of a coronavirus with a 27 kb-long RNA genome from a full-length genomic copy. Another attractive feature is that the approach may overcome a limit on a packaging capacity of viral particles. Alphaviral genomes naturally have lengths 11-12 kb and the maximal limit of the packaging capacity of the alphavirus virion was reported to be ~13-14 kb, leaving only ~2 kb for heterologous inserts [8]. The alphavirus replicase itself can replicate significantly larger templates, at least 32 kb-long [9], although such long genomes are supposed to be inefficiently packaged. Very long RNA constructs capabile of autonomous replication in the cytoplasm can be produced utilizing DNA-launched infectious clones.

By their plasmid nature and presence of eukaryotic transcriptional promoters, DNA-launched clones resemble common eukaryotic expression vectors, e.g. that contains cloned cDNAs under the promoters≥ transcriptional control. Other genetic elements common to both types are transcriptional termination/polyadenylation signals. In general, expression levels of artificial genes created from cDNA are low (in some published examples, lower by several hundred times) than the expression levels for the same proteins from their natural genes [10-14]. The difference in the expression levels is largely because natural eukaryotic genes contain introns whereas cDNA-derived artificial genes do not contain introns. It is known for long time, that introns are not mere flnon-functionalffi DNA, rather the introns play numerous roles in the processes of transcriptional initiation and elongation [15, 16], modulating polyadenylation efficiency [17,18], controlling stability of mRNA [19], and directing nuclear export [20, 21]. Introns are removed from primary transcripts by a process termed splicing. Each splicing event leaves a mark on mRNA in the form of the exon-junction complex (EJC) positioned in proximity to a splice site. The EJC proteins remain associated with spliced mRNA during and after the nuclear export. In the cytoplasm, the EJC-labeled mRNA undergoes increased translation [22]. Expression augmentation by introns is so extreme in plants that this phenomenon was designated with the special term: intron-mediated enhancement (IME) [23]. However the IME is not a property of only the plant world, as it has been demonstrated in animal models [11, 22]. Including introns in transcribed sequences within expression constructs helps achieving higher expression levels

[24]. With regards to engineering of the IME, it has a positional effect: IME magnitude diminishes with increasing distance (of an intron position) from a transcription start site.

Frequently the first (promoter-proximal) intron in a gene is involved in the IME [14, 15, 25-28]. In the cited examples, the first introns harbor signals of transcription initiation and thus contribute to the IME. Often, the promoter-proximal exons are noncoding (i.e. they map to 5≥UTRs in transcripts). Also, the promoter-proximal introns differ from other introns in same genes by a nucleotide composition and length (the promoter-proximal introns are longer than downstream introns) [29].

The IME utilization in DNA-launched molecular clones may be beneficial. Introns have been inserted in molecular clones of RNA-viruses mainly for a purpose of stabilization of inserts during propagation of these plasmids in *E.coli* [30]. It was postulated that sequences resembling bacterial promoters (i.e. cryptic promoters) are present in genomes of RNA-viruses. These cryptic promoters drive abnormal transcription from viral cDNAs that leads to the generation of aberrant mRNAs. Translation of the aberrant mRNAs results in appearance of viral proteins that appear to be toxic to *E.coli* [31-33]. With regard to the unwanted during propagation of the molecular clones in bacteria, it is of interest that the CMV promoter itself has a transcriptional activity in *E.coli* [34]. Toxicity of the aberrant expression products leads to rearrangements in cDNA regions. Cloning of an intron into the cDNA region interrupts the main ORF and prevents the rearrangements [30-37]. It is expected, that the intron(s) engineered in a viral genome will be removed from the primary transcripts during RNA-splicing and the mature transcripts will be capable of autonomous replication.

Currently, a number of DNA-launched infectious clones of alphaviruses have been developed for possible use as vaccines, although few of the published constructs contain engineered introns [37]. In published examples from one research group, an intron belonging to a specific type (capable of splicing regulation by addition of antisense oligonucleotides) was used to control virus rescuing efficiency. Although, in the mentioned examples, presence of the intron actually diminished the virus rescue efficiency.

In this study, we investigated the efficiency of VEE rescuing from a DNA-launched infectious clone that has an intron engineered in proximity to the translational start site.

Materials and methods

Cell cultures. Baby Hamster Kidney (BHK-21) are from a collection at the National center for biotechnology (Nur-Sultan, Kazakhstan). The cells were grown in a complete medium (DMEM with high glucose (Lonza BE12-604F/U1) supplemented with 10% fetal calf serum (FBS, Gibco 16000-044), 1% penicillin/streptomycin, 2 mM L-glutamine) at 37°C in atmosphere of 5% CO_2.

Genetic engineering. Enzymes were purchased from ThermoFisher Scientific. Standard methods of genetic engineering were used to manipulate DNA. Molecular designs of the DNA-launched infectious clones are shown in Figure 1.

A cDNA copy of the VEE genome is from strain TC-83 (Genbank: L01443), except mutations reducing viral cytopathicity were introduced in the viral nSP2 and C genes as described in [38]. The virus expresses GFP during intracellular replication. A GFP gene is cloned downstream the second (engineered) copy of the SGp. The viral genome is cloned in an eukaryotic expression plasmid under the control of the CMV immediate-early promoter. An assembly of the delta-virus antigenomic ribozyme and the polyadenylation signal of the bovine growth hormone gene is cloned downstream of the genomic 3≥-end. The resulting plasmid pCMV-VEE-GFP is deposited to Genbank (accession number MH891622).

The DNA-launched infectious clone pCMV-VEE/insPst was produced from pCMV-VEE-GFP by inserting the 7 nt-long sequence CTGCAGG in the viral genome immediately downstream the 5≥UTR. This inserted sequence is a combination of the *PstI* restriction site and the consensus splice acceptor site (AS). The consensus acceptor site for the major class (U2 snRNA-dependent) introns is CAGG.

The DNA-launched infectious clone pCMV-VEE/Intron has an intron in the viral genome between the 5≥UTR and the nsP1 gene. The intron is the β-globin/IgG chimeric intron present in pCI/pSI families of mammalian expression plasmids developed by Promega. The intron sequence was synthesized *de novo* with flanking PstI sites and ligated in the PstI site located in pCMV-VEE/insPst.

The plasmids (DNA-launched infectious clones) were prepared using an alkaline lysis method, purified by banding in a cesium chloride gradient and used for transfections.

Transfections. BHK-21 cultures were grown to ~90% confluence before electroporation. Cells were harvested by trypsinization and collected by centrifugation at 1000 rpm for 5 min at 0°C. The following procedures were done at 0°C, using an ice-cold electroporation buffer. The cell pellet was resuspended in 7 ml of PBS (Sigma D8537) and the cells were collected again. One more wash with the same electroporation buffer was performed as described. The final cell pellet was re-suspended in PBS and the cell suspension was diluted to obtain $1x10^7$ cells in a 400 ul aliquot. The aliquot (400 ul) was mixed with 10 ug of a plasmid. The mixture was transferred to a chilled electroporation cuvette (2-mm gap, Sigma Z706086) and two exponential-waveform pulses were done with 1500V, 25 uF capacitance and infinite resistance. The cells were left to recover for 10 min at room temperature. The electroporated cells were either used in an infectious centers assay or plated in a culture to produce a virus growth curve.

Infectious centers assay, limiting dilution (Reed-Muench) method. Before starting of the electroporation experiments, 96-well plates were seeded with BHK-21 cells ($3.75x10^4$ cells per well) and the cells were allowed to attach (~4 hours).

An aliquot of electroporated cells (0.4 ml) was mixed with 1.6 ml of the complete medium and further the sample was used to produce serial dilutions (from 1:10 to $1:10^7$) in the complete medium. The medium was removed from 96-well plates with growing BHK-21 monolayers. Portions (0.1 ml) of the serial dilutions were distributed in the wells. A long row (12 wells) of the plate was used to distribute a particular dilution. The plates were incubated in 5% CO_2 at 37°C and monitored for at least 3 days for GFP fluorescence. A fraction of the wells with infected monolayers was counted for each row. Focus-forming units (FFU) were calculated using the Reed-Muench method and equation [39]. The efficiency of virus rescue is expressed as the FFU counts per 1 ug of the electroporated plasmid.

Virus production and building growth curves. An aliquot of electroporated cells (0.4 ml) was mixed with 10 ml of the complete medium and seeded in a P100 dish (TPP 93100). The medium on the transfected culture was completely replaced every day with the fresh medium. The spent medium (virus sample) was collected every day to determine virus titers. 6-well plates with growing BHK-21 monolayers were prepared by seeding of $5x10^6$ BHK-21 cells per well and allowing the cells to attach. For titration, serial dilutions ($1:10^4$ -$1:10^9$) were prepared by diluting of the virus samples in the PBS with 1% FBS. Medium was removed from wells of the 6-well plates and aliquots (0,2 ml) of the dilutions were distributed in the wells. The plates were incubated for 1 hour in 5% CO_2 at 37°C with occasional shaking. Infectious inocula were removed from the wells and the monolayers were covered with molten agar-containing growth medium (DMEM with 3.3% FBS, antibiotics and 0.5% agar). Agar medium was allowed to solidify and the plates were incubated for 3 days and observed for GFP fluorescence. Foci of GFP-producing cells were counted and the numbers were recalculated in virus titers.

Statistical analysis. All experiments were performed in triplicate. Mean values were calculated and ranges between the highest and lowest values were recorded. During analysis of growth curves, titers corresponding to the stationary phase of an infection (days 2-5 p.i.) were compared. Statistical significance in pair wise comparisons was assessed by Mann-Whitney test using GraphPad Prism 6.0 (GraphPad). Differences were considered statistically significant when $p<0,05$.

RESULTS

The initial DNA-launched infectious clone. The plasmid pCMV-VEE-GFP contains a cDNA-copy of the VEE genome placed under the control of the CMV promoter. Hepatitis D virus (HDV) antigenomic ribozyme (RBZ) and human growth hormone (HGH) polyadenylation signal are placed downstream the genome≥s 3≥-end (figure 1).

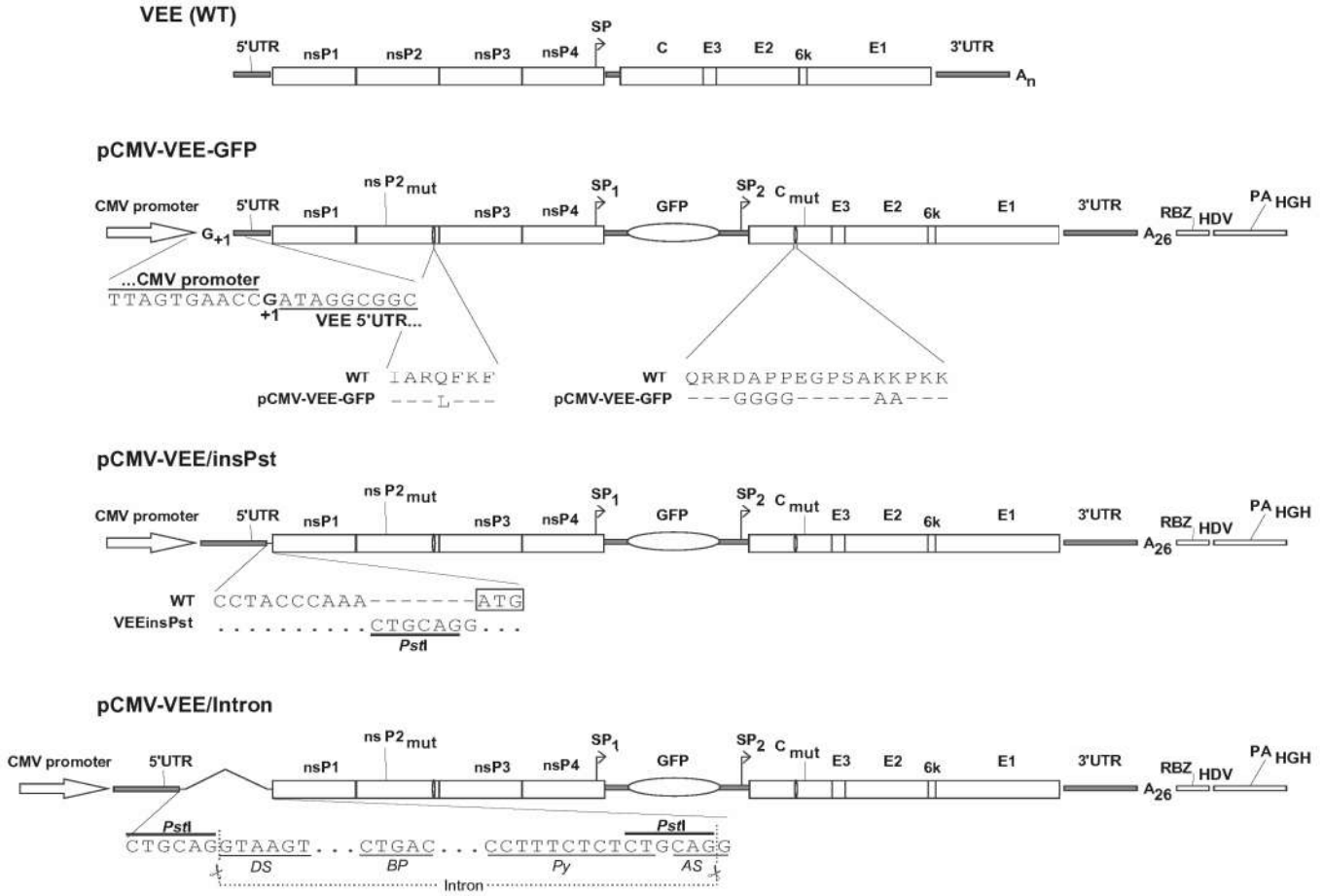


Fig. 1. Genetic designs of DNA-launched infectious clones

Panel VEE (WT), a schematic representation of the VEE genome. A wild type (WT) is the TC-83 strain. Genes for viral proteins are shown as open rectangles. Untranslated regions are depicted as solid lines. The arrow designates a subgenomic promoter (SGp). The VEE genome terminates with a polyA tail (An).

Panel pCMV-VEE-GFP, a part of a plasmid containing the viral genome. The CMV promoter≥s transcriptional start site (G+1) precedes the first nucleotide in VEE 5≥UTR (sequence of the junction region is shown). The genome≥s 3≥-end is a stretch of 26 adenine residues (A26). The hepatitis D virus ribozyme (RBZHDV) and the HGH polyA signal (PAHGH) are placed after the 3≥-end of the genome. The VEE genome bears cytopathicity-reducing mutations in the nsP2 (nsP2mut) and capsid (Cmut) genes (positions of the mutations are depicted). Comparisons of aminoacid sequences below the genetic map show the wild type (WT) and mutant sequences (nsP2mut or Cmut). The cloned genome bears two subgenomic promoters (SP1, SP2): RNA transcribed from the SP1 is translated to produce GFP (depicted as the ellipse figure); RNA transcribed from the SP2 is translated to produce the viral structural proteins.

Panel pCMV-VEE/insPst, the clone in which the VEE genome is modified by inserting CTGCAGG sequence between the 3≥-end of the 5≥UTR and the beginning of the nsP1 gene. The CTGCAG sequence is the PstI restriction site. A comparison of the 5≥UTR-nsP1 junction region in the wild type virus and assembled construct is shown below the genetic map. The translational initiation codon (ATG) is enclosed in the box.

Panel pCMV-VEE/Intron, the clone in which an intron is inserted just after the VEE 5≥UTR. The intron (depicted as an angle shape) is flanked with PstI sites. The used intron is a hybrid of the 2-nd b-globin intron and the IgG intron. Sequences of the donor site (DS), branch point (PB), polypyrimidine stretch (Py) and acceptor site (AS) are shown. Borders of the intron are marked by the scissors signs.

A sequence of pCMV-VEE-GFP is deposited to Genbank (MH891622). The VEE genome is from TC-83 strain with cytopathicity-reducing mutations engineered in nsP2 and capsid protein genes. To provide for formation of the functional 5≥-end of virus RNA during transcription from the CMV promoter, its preferred transcriptional start nucleotide (G+1) was placed immediately preceding the first nucleo-

tide in the 5≥UTR.

The virus genomes in all DNA-launched infectious clones produced in this study have mutations to make replication of the viruses non-cytopathic. The mutation described in [40] results in a replacement of one aminoacid residue (Gln739->Leu) in the nonstructural protein nsP2. This mutation makes the replication of VEE replicons (fragments of VEE RNA without structural genes) non-cytopathic. Although this mutation alone is not sufficient for the noncytopathic replication of the virus which has native genes for the structural proteins. Clusters of mutations were introduced in the gene encoding capsid (C) protein. The latter mutations abolish ability of the capsid protein to interfere with nuclear export, so that the mutant capsid protein is no more capable of installing transcriptional and translational shut-off in virus-infected cells.

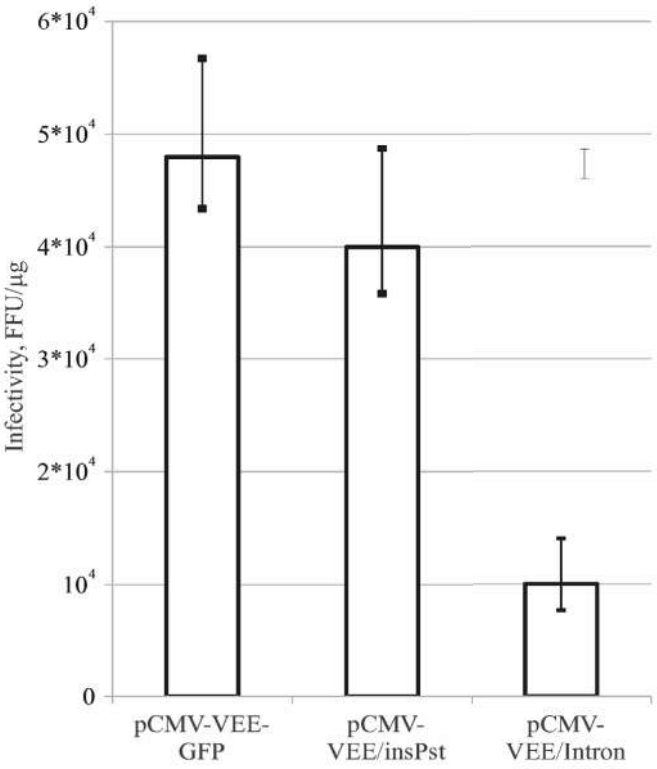
In all DNA-launched infectious clones, the antigenomic ribozyme of hepatitis D virus (HDV RBZ) is placed downstream of the oligo-A stretch terminating the VEE genome. Following the RBZ, a polyadenylation signal of the human growth hormone (HGH) gene is present. Primary transcripts are expected to be cleaved by the HDV RBZ to produce the correct 3≥-termini [7].

A GFP gene is present in virus genomes in all clones. Assembly of a GFP-expressing VEE replicon has been described in [38]. Fragments representing the SGp and sgRNA≥s 5≥UTR were added to the 5≥-end of the GFP gene and the extended gene was cloned upstream of the ORF for structural proteins (Figure 1). Thus, the viruses in this study have two copies of the SGp: the first copy (SP1) drives synthesis of a type of sgRNA which is translated to produce GFP; the second copy (SP2) controls the generation of the second type of sgRNA encoding the structural proteins C-E3-E2-6k-E1.

Intron inserted in the VEE infectious clone. The construct pCMV-VEE/insPst is different from pCMV-VEE-GFP by presence of the nonnative sequence CTGCAGG between the 5≥UTR and the nsP1 gene. This DNA-launched infectious clone was constructed to check the ability of VEE to tolerate insertions in this region (downstream the 5≥UTR) and to measure influence of nonnative sequences in this position on viral vitality. The inserted sequence contains the PstI restriction site which is convenient for cloning of various major-class (U2 snRNA-dependent). The U2-dependent introns are the most prevalent class of introns in genomes of higher eukaryotes [41] and are used in constructs for recombinant expression [11, 24, 42, 43,44].

The viral genome in pCMV-VEE/Intron is interrupted with the chimeric (β-globin/IgG) intron present between the 5≥UTR and the nsP1 gene. The same intron is used in the pCI/pSI family of mammalian expression vectors (Promega). The same intron was successfully utilized to achieve high expression levels using adeno-associated virus (AAV) as the expression vector.

Presence of the intron has a small impact on rescuing efficiency and no effects on maximal titers. Results of measuring of rescue efficiency (DNA-infectivity) for three DNA-launched infectious clones are presented in figure 2.



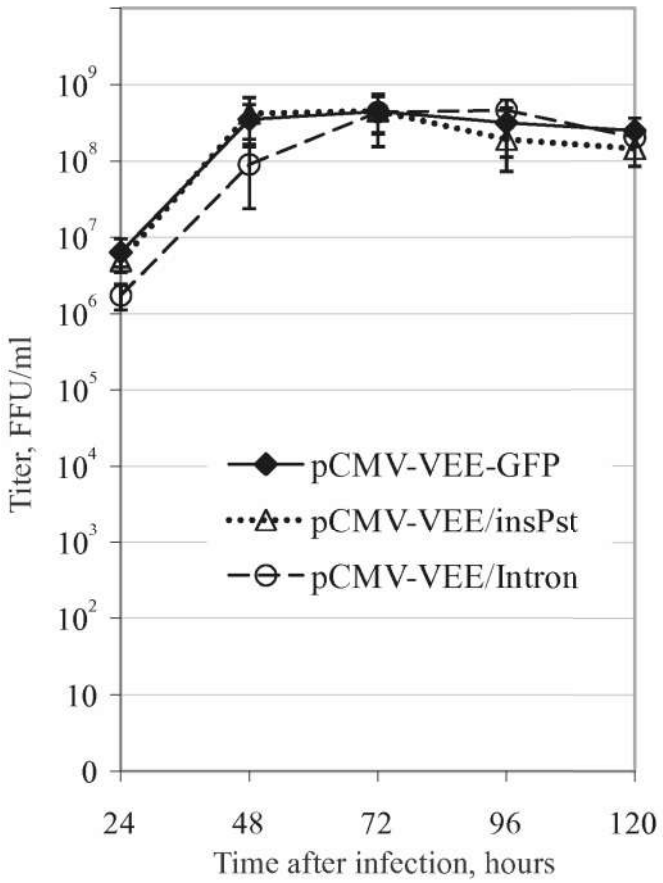
The efficiency is expressed as a number of foci (FFU) produced after transfecting of 1 ug of a corresponding plasmid. Bars heights show mean values. Whiskers show a range between the maximal and minimal values (FFU/ug) produced in three experiments.

Fig. 2. Rescuing efficiency for the DNA-launched infectious clones pCMV-VEE-GFP, pCMV-VEE/insPst and pCMV-VEE/Intron

Mean values for numbers of foci produced after transfecting of 1 ug of a plasmid are $4{,}8x10^4$ (FFU) for the parental construct (pCMV-VEE-GFP), $4{,}0x10^4$ for the construct with the inserted PstI site (pCMV-VEE/insPst), and $1{,}0x10^4$ for the construct with the intron (pCMV-VEE/Intron). A difference in the DNA-infectivity for the plasmids pCMV-VEE-GFP and pCMV-VEE/insPst is not statistically

significant (p>0,999). However, there is a statistically significant difference between the results for pCMV-VEE-GFP and pCMV-VEE/Intron (p=0,001). Thus, insertion of the intron in the selected genomic position reduces the DNA-infectivity, although a magnitude of the reduction is not large (4,8 times) in comparison to known variability resulting from varying experimental conditions during virus rescuing experiments.

Growth curves for viruses rescued from the DNA-launched molecular clones pCMV-VEE-GFP, pCMV-VEE/insPst and pCMV-VEE/Intron are shown in Figure 3.



Titers are expressed as focus-forming units (FFU) in 1 ml. The curves show mean values. Whiskers show a range between the maximal and minimal titer for each time point produced in three experiments.

Fig. 3. Growth curves for viruses rescued from the DNA-launched molecular clones pCMV-VEE-GFP, pCMV-VEE/insPst and pCMV-VEE/Intron

The viruses pCMV-VEE-GFP (parental) and pCMV-VEE/insPst (with the inserted PstI site) are practically identical in growth behaviors and their stationary titers are not different (p>0,999). Also, there is no statistically significant differences in stationary-phase titers for pCMV-VEE-GFP and pCMV-VEE/Intron (p>0,999). The latter result is expected because the intron is removed from primary transcripts and this splicing event generates mature transcripts which are designed to be genetically identical to pCMV-VEE/insPst.

DISCUSSION

Two techniques are commonly used to rescue live viruses from the infectious (molecular) clones. The first technique (naked RNA) relies on cloning of a cDNA-copy of a virus genome under transcriptional control of a small bacteriophage-derived promoter which is suitable for in vitro transcription. Viral RNA is produced in vitro with the use of a phage-derived RNA polymerase and this RNA is transfected in cell cultures or may be injected in animals [45]. The second technique has several names, including infectious DNA (iDNA) [46], or DNA/RNA-layered system, or DNA-launched virus [47]. All these names refer to the technique when a cDNA-copy is placed in a plasmid downstream of an eukaryotic promoter, e.g. cytomegalovirus (CMV) immediate-early promoter. Upon delivery of a DNA-launched clone into a cell, a fraction of the transfected DNA is transported to the nucleus where the strong RNA polymerase II promoter drives production of primary RNA transcripts which can further undergo maturation. Subsequently, the transcripts are transported to the cytoplasm where they initiate the autonomous replication.

Using of DNA-launched clones allow circumventing some limitations inherent to the naked RNA-transfection approach such as a limit on a length of RNA which can be produced in vitro. The most efficient protocols for in vitro transcription allow producing ~15 kb-long RNA [4]. The alphavirus replicative machinery is capable of replicating significantly longer genomes [9]. This means that the naked RNA approach cannot fully explore possible scope of applications of the alphavirus vectors, whereas DNA-launched clones can hold inserts of practically unlimited size.

Inserting of introns in otherwise intron-less genes was demonstrated to increase genes≥ expression [23], affecting among others transcription initiation [98], transcript≥s 3′-end processing and mRNA stabilization [48], nuclear export, and even efficiency of post-nuclear processes (mRNA translation) [22]. Inserting of an intron into a cloned cDNA of a cellular gene sometimes increases its expression up to 300 times [49] and to 900 times.

Development of molecular clones for alphavi-

ruses may require selection of a vector, a host strain and additional engineering of plasmids to circumvent instability and toxicity during their propagation in *E.coli*. A reason for these effects is believed to be that bacterial promoter-like sequences are present in viral genomes, and the cryptic promoters drive transcription of mRNA (aberrant mRNA). In turn, translation of the aberrant mRNAs leads to accumulation of virus-encoded proteins toxic to *E.coli* [31-33]. The human cytomegalovirus (CMV) immediate-early promoter commonly used in DNA-launched constructs is also active in *E.coli* [34].

Improvement of stability of molecular clones during propagation in*E.coli* was achieved by engineering of one or more artificial introns in the cloned cDNAs to interrupt a viral ORF. The engineered introns interrupt the main ORF by presenting translational stop codons and by frame shifting; the result is premature termination of translation from the aberrant mRNAs. Efficiency of introns for the purpose of stabilization of molecular clones has multiple proves in regard to the alphaviruses [30, 50] as well as to cDNA clones of many other RNA-viruses [33].

Unexpectedly the authors of this study failed to find published literature which describes cloning of introns into viral cDNA constructs for purposes other than the plasmid stabilization.

In this study we demonstrate the creation of a DNA-launched clone for VEE genome and compare efficiency of rescue for native and intron-containing genomes.

Acknowledgments

This work was supported by the Ministry of Education and Science of Kazakhstan (grant No. AP05130607.)

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ҚОЛДАНБАЛЫ ИНТРОНЫ БАР ДНҚ-БАСТАҒАН ЖҰҚПАЛЫ КЛОН: ВИРУСТЫҢ ӨСУІ МЕН ҚҰТҚАРЫЛУЫНА ӘСЕРІ

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ТҮЙІН

Венесуэлалық жылқы-энцефалиты вирусына (VEE) арналған ДНҚ-бастаушы жұқпалы клондар алынды. ДНҚ-бастаушы жұқпалы клон - бұл эукариот промоторының бақылауында орналасқан вирустық геномның кДНҚ толық геномды көшірмесі бар плазмида. Плазмиданы жасуша культурасына трансфекциялаудан кейін вирус құтқарылатын болды. Әзірленген конструкцияларда VEE геномының кДНҚ-кшірмесі цитомегаловирус (CMV) промоторының бақылауында болды. Рибозим және полиаденилдау сигналы геномның 3' соңынан кейін инженирленді. Вирустың құтқарылу тиімділігі мен өсу кинетикасына ДНҚ-бастаған инфекциялық клонның интронға инсерциясының ықтимал әсерін тексеру үшін кішігіім гибридті интрон (2-ші b-глобиндік интроннан және IgG интронынан құралған) вирустық 5'UTR мен nsP2 генінің арасына клондалды. Барлық конструкциялар үшін құтқару тиімділігі жоғары болды: ата-аналық конструкция үшін трансфекцияланған плазмидты ДНҚ-ның 1 мкг-на 4,8x104 фокус құрушы бірлігі (FFU) сәйкес келді, UTR және nsP2 генінің арасындғы PstI-дің табиғи емес учаскесі үшін 4,0x104 FFU/мкг және 1,0x104 FFU/мкг интроны таңдалған геномдық позицияға орналастыруға арналған болды. Құтқарылған вирустар инфекцияның стационарлық кезеңіне ұқсас титрлерге ие болды. Осы зерттеуде біз PstI сайттарына табиғи немесе инженирленген интрондарды орналастырудың тиімді әдісін көрсетеміз.

Негізгі сөздер: альфавирус, венесуэлалық жылқы энцефалиті вирусы, ДНҚ-бастаушы жұқпалы клон, интрон, вирусты құтқару, CMV промоторы.

ИНФЕКЦИОННЫЙ МОЛЕКУЛЯРНЫЙ ВИРУСНЫЙ КЛОН С ИСКУССТВЕННЫМ ИНТРОНОМ: ВЛИЯНИЕ НА ОЖИВЛЕНИЕ И РОСТ ВИРУСА

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АБСТРАКТ

Созданы инфекционные клоны вируса венесуэльского энцефалита (VEE), позволяющие оживлять вирус путём трансфекции плазмидных ДНК. Инфекционный молекулярный вирусный клон представляет собой плазмиду, содержащую кДНК-копию полного вирусного генома; кДНК находится под контролем эукариотического промотора. В исследованных конструкциях кДНК-копия генома VEE находится под контролем промотора цитомегаловируса человека (CMV). Рибозим и сигнал полиаденилирования встроены за 3'-концом вирусного генома.

В данной работе было изучено влияние встройки искусственного интрона в вирусную кДНК на эффективность оживления вируса и возможное влияние на кинетику роста. Небольшой гибридный интрон (состоящий из фрагментов 2-го интрона b-глобина и интрона гена IgG) был клонирован между областью 5'UTR и геном nsP2.

Эффективность оживления оказалась высокой для всех конструкций: 4,8 × 104 фокус-образующих единиц (FFU) на 1 мкг плазмидной ДНК для родительской конструкции (без вставок), 4,0 × 104 FFU/мкг для конструкции со вставкой сайта PstI между 5'UTR и nsP2, и 1,0 × 104 FFU/мкг для конструкции с интроном. Вирусы, оживлённые из трёх конструкций, достигают эквивалентных титров. В этом исследовании показан эффективный метод встраивания интронов в сайты PstI, которые могут быть природными или искусственно созданными.

Ключевые слова: альфавирус, вирус венесуэльского энцефалита, инфекционный молекулярный клон, интрон, оживление вируса, CMV промотор