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THE STUDY OF FACTORS AFFECTED THE GENE TRANSFER EFFICIENCY IN CHICKEN EMBRYONIC CELLS BY APPLICATION OF LENTIVIRAL VECTORS

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Abstract

Lentivirus-mediated gene transfer is being the one of the attractive method for genetic modification of chicken (S.C. Chapman et al., 2005; C.A. Smith et al., 2009; N.A. Volkova et al., 2013). However, the efficiency of thansgenesis of the chicken embryonic cells has been shown to be relatively low. Therefore, a large number (60,000 to 100,000) of embryonic cells at the start of incubation and the virus preparations with high titers (about 10^9 particles per milliliter) remain one of a crucial problem the researchers are facing with when try to achieve a satisfactory transgene introduction. The aim of the present study was to determine the optimal conditions for production and application of the modified lentiviral vector system of second generation for the transgenesis of chicken embryos. The vector system consisted of three different plasmids: psPAX2, containing gag-pol genes; pLPG, coding envelop glycoprotein G of vesicular stomatitis virus (VVC-G) and pWPXL, the self-inactivated lentiviral vector, carrying *eGFP* (enhanced green fluorescence protein) gene under control of promoter region of the human elongation factor 1 alpha-encoding gene. To produce the recombinant virus particles and to determine the virus titers we used human cell line 293T. The injections of the virus preparations into the chicken embryos were performed at the different stages: from 20 to 24 hours (group 1) and from 50 to 55 hours (group 2) of incubation. To detect the transgenesis efficiency and the number of the integrated copies of the transgene the total DNA was extracted from embryos on the day 7 of incubation and analyzed for the presence of specific *eGFP* sequences by real-time PCR. The maximal titers of the virus preparations were produced by the ratio of the psPAX2, pLPG and pWCAG plasmids equal 1:1:3 and were 2.4×10^7 CFU/ml before ultracentrifugation and 6.2×10^8 CFU/ml after concentration by ultracentrifugation. The efficiency of genetic transformation which was evaluated as the part of the transformed cells from the overall number of analyzed cells was 78.0 and 31.0 % in the groups 1 and 2, respectively. It was shown, that the alteration in the ratios between components of the vector system comparing to the standard scheme allows significantly increase the titers of the produced virus preparations. The biological titers of the virus preparations of 10^8 CFU/ml are sufficient to infect up to eighty percent of cells at the earlier stages of embryo development. Presumably, at earlier embryogenesis the cells were infected with more viral particles resulting in different number of the transgene copies integrated into cell genome in the groups 1 and 2. From the obtained results, the efficiency of transgenesis by means of the lentiviral vectors ranged from 30.0 to 34.3 % and varied slightly depending on time after the embryos incubation began. These results indicate that only a part of embryonic cells is usually available for viral infection. Injection of embryos at different intervals of incubation by viral preparations with similar titers produced the populations of embryonic cells with different amounts of vector copies in the cell genome. So the efficiency of hen embryo thransgenesis does not depend on the stage of its development at least for 55 hours of the embryogenesis and can be predictable.

Keywords: lentiviral vectors, molecular cloning, transfection, transgenic animals.

By now, the prospects of the use of retroviral and lentiviral vector sys-

tems for genetic modification in poultry has been shown in several studies [1-6]. Therefore, a large number (60,000 to 100,000) of embryonic cells at the start of incubation and the need for virus preparations with high titers (about 10^9 particles per ml) remains one of the crucial problems the researchers are facing when trying to achieve satisfactory transgene introduction. The use of constitutive promoter enhancers such as promoter enhance of early human cytomegalovirus genes (CMV) and promoter enhancer of bird β-actin gene makes it possible to achieve significant expression (up to a few milligrams of protein per 1 ml of serum and egg protein) in mosaic form (G_0) obtained from embryos after transgene introduction. However, expression is significantly reduced in next generations (G_1 and G_2) [5]. The reasons for this are not understood. Probably, high expression of a foreign protein acts as a selective factor in various organs and tissues. Furthermore, it has been noted that the expression level is directly correlated with the gene dose (number of viral copies per cell genome), i.e. the possibility of various physiological defects in the most promising producers is greatly increased [2, 7-9]. Attempts were also made to achieve tissue-specific transgene expression using regulatory elements that control the synthesis of egg ovalbumin [10-14]. According to the authors, expression of transgenes in several generations of animals was relatively stable, yet the reached levels of protein synthesis were approximately 20-50 times lower than when using constitutive promoters.

Vector systems derived from integrative viruses such as lenti- and retroviruses are an effective tool for the introduction and expression of genes due to a number of unique features: the members of the family are able to stably integrate into the genome of the host cell; relatively small genome sizes allow them to be easily manipulated *in vitro*; the internal genome sequences can be removed in such a way that all the functions required for replication are provided in trans; using surface virus glycoproteins that are tropic to a broad host range, it is possible to infect almost any species and type of vertebrate cells with hybrid virions.

The first stage of our study of the features of gene introduction and expression was to optimize the standard protocol of vector production based on lentiviral system, which made it possible to significantly increase the titer of the viral preparation used. The findings of our study of the conditions of chick embryo infection with lentiviral preparations give grounds to suppose the predictability of the result.

The purpose of the research presented was to evaluate the effect of the number and proportion of the lentiviral vector system components on physical and biological titers of the virus produced, as well as of the time of introduction of the resulting lentiviral vector in chick embryos on the effectiveness of transgenesis in vivo.

Technique. The research was made with the chicks of the Ptichnoe cross. We used a modified lentiviral vector system of the second generation which included three different plasmids, psPAX2, containing *gag-pol* genes, pLPG encoding surface glycoprotein G of vesicular stomatitis virus (VVC-G), and pWPXL, the self-inactivated lentiviral vector, carrying the *eGFP* (enhanced green fluorescence protein) gene under the control of human PNA-polymerase II elongation factor 1 (hEF1α) [15]. The pWPXL plasmid was used to construct the pWCAG vector. Viral vector was constructed using standard molecular cloning techniques [16].

To produce recombinant virus particles and to determine virus titers, we used the 293T human cell line. Cells of this line have the ability to divide rapidly and can be transfected with plasmid DNA with high efficiency. Cells were cultured in the DMEM medium (Dulbecco's Modified Eagle's Medium) containing fetal calf serum (10 %), L-glutamine (2 mM), penicillin (100 U/ml), strep-

tomycin (100 μg/ml) in the atmosphere of 5 % CO_2 at 37 °C. Lentiviral vector was introduced into the 293T cell line by calcium phosphate precipitation [16]. At the same time, 1.7×10^6 cells were placed in a vial with substrate area of 25 cm^2 for 1 day before the start of the experiment. At day 2, the culture medium was replaced with 1 ml of fresh medium containing DNA of all three plasmids of the vector system in different ratios and incubated for 6 hours in the presence of calcium phosphate buffer. Then the cells were chemically shocked by treatment with 20 % DMSO (dimethyl sulfoxide) for 5 minutes, then washed and placed in fresh medium. Next day, the culture medium was replaced with fresh media and incubation was continued for 48 hours, after which the culture supernatant containing virus was collected, clarified by centrifugation and aliquots were frozen at –80 °C. For concentrating virus preparations, culture supernatants were ultracentrifuged (70,000 g, 120 min, +4 °C) and pellets were resuspended at +4 °C in a small volume of TNE buffer (50 mM Tris-HCl, pH 7.8, 130 mM NaCl, 1 mM Na_2-EDTA).

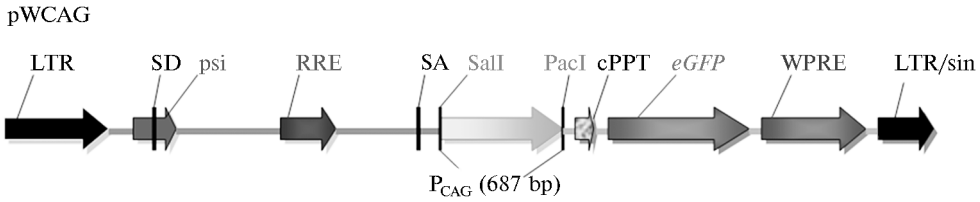
To determine the physical titer of aliquot viral vectors (5-50 μl) of culture supernatants containing the virus, viral RNA was isolated using a Qiagen (USA) kit. Viral RNA served as matrix for cDNA synthesis with reverse primer specific for the *eGFP* gene sequences. Then, the number of vectors in the real-time PCR (RT-PCR) was determined using cDNA synthesized and primers specific to the *eGFP* gene. A series of 2-fold dilutions of the viral vector plasmid DNA in the desired range was the standard for evaluating the number of gene copies. RT-PCR was conducted using a MiniOpticon™ apparatus (Bio-Rad, USA). Biological virus titer was determined in accordance with the description given by G. Tiscornia et al. [17]. The supernatant collected at culturing 293T cell clones transfected with retroviral vectors was centrifuged to remove cells and debris (10 min, 3,000 g). Infectioning was conducted immediately after virus collection. Recipient 293T cells were planted at a density of 3×10^5 in Petri dishes 60 mm in diameter 1 day prior to infecting. At day 2 the medium was replaced with 1 ml of fresh medium containing polybrene (8 mcg/ml) and aliquots of test virus. After 6 hour incubation, medium was changed by fresh one. After 48-72 hour incubation, cells were detached from the substrate with trypsin, resuspended in PBS and EGFP fluorescence intensity was measured using a flow cytometer FACSCanto (BD, USA) with a set of 480-490 nm (excitation) and 510 nm (emission) filters.

Chromosomal DNA was isolated from chick embryos by salt method [16]. DNA concentration was measured spectrophotometrically (Hitachi U-1100, Japan) at $\lambda = 260$ nm ($OE_{260} = 1$ corresponds to 50 μg of double-stranded DNA). The purity of DNA preparations was evaluated by calculating the ratio of optical density at wavelengths of 260 nm and 280 nm. Normalization of DNA samples was performed using RT-PCR with primers and hybridization probe specific to DNA encoding 18S rRNA in *Gallus gallus*. The number of DNA vector copies was determined with primers and hybridization probes specific of *eGFP*. To prepare standards, we used plasmid viral vector DNA transferred into the linear shape at a unique restriction site titred in a previously determined range. PCR-products were produced in the reaction mixture of 25 μl containing 1× buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.08 % Nonidet P400), 2 mM $MgCl_2$, 0.2 mM dNTPs, primers (0.25 μM for each), hybridization probe (0.125 μM), Taq-polymerase (1.25 U, Fermentas, Lithuania), and 5 ng of matrix. Amplification conditions were 95 °C, 5 min; 45 cycles of 94 °C, 30 s; 50 °C (varies), 30 s; and 72 °C, 1 min. Quantitative RT-PCR and experimental selection of optimal oligonucleotide annealing temperatures was also performed using a MiniOpticon™ unit (Bio-Rad, USA).

The analysis of eGFP expression in embryo tissues was performed using a flow cytometer (FACSCanto, BD, USA).

Results. We used a lentiviral vector system to determine the conditions for the effective introduction of transgenes in embryonic chicken cells. In particular, the modified system of the second generation was chosen which, as already mentioned, included three different plasmids: the first one encoded the vesicular stomatitis virus (VVS-G) surface glycoprotein G, the second one was a classic «packager» and contained the *gag* and *pol* genes, the third one was a self-inactivated vector (SN, self-inactivated) [15].

To study the efficiency of introduction of gene expression in poultry cells, the *eGFP* gene was used as a structural and hybrid CAG enhancer promoter of early human cytomegalovirus genes (hCMV, human cytomegalovirus) promoter and chicken β-actin promoter. A construct with the eGFP gene under the control of CAG promoter was produced using these sequences based on self-inactivated lentiviral vector genome.



Structure lentiviral expression vector pWCAG. Description of the viral vector construction is given in the text. LTR, LTR/sin (LTR, long terminal repeat; sin, self-inactivating), lentiviral long terminal repeats: 5′-LTR — wild type, 3′-LTR/sin — self-inactivating version. SD, SA (splice donor, splice acceptor) — donor and acceptor splicing sites; psi — region responsible for packaging of viral genomic RNA into virion; RRE (rev responsible element) — site of binding of Rev-protein responsible for the transportation of genomic RNA molecule from nucleus to cytoplasm; cPPT (central polypurine tract) — central polypurine tract involved in transportation of pre-integrative complex to cell nucleus; *eGFP* — enhanced green fluorescence protein gene; WPRE — woodchuck post-transcriptional regulatory element. P_{CAG} — hybrid regulatory element containing early human cytomegalovirus gene enhancer and chicken β-actin gene promoter. SalI, PacI — restriction sites used to clone regulatory elements.

The structure of lentiviral vector is shown in the figure. To obtain pWCAG vector, a fragment of 687 bp with the hybrid CAG enhancer-promoter contained in the psPAX2 plasmid [13] was amplified, gel purified and cloned by SalI-PacI restriction sites as part of the pWPXL plasmid (15).

The inter-component ratio in transfection was optimized experimentally.

As already mentioned, in production of viral preparation, plasmids psPAX2, pLPG, and pWCAG forming a ternary vector system, were introduced into human cells (line 293T) by $CaPO_4$ precipitation. Using plasmids in different proportions and changing their total number (while maintaining the proportion) and medium composition, conditions were selected to ensure the high titer output of viral particles.

Table 1 summarizes the data illustrating the effect of different ratios between the components of the vector system on physical and biological virus titer. As is evident, a 2-fold decrease or increase in the number of all three plasmids compared to the original version did not have a significant impact on the viral titer (change of no more than 2 times). At the same time, an increase in the packer (pLPG) and vector (pWCAG) amount compared to the standard version (1:2:3, psPAX2:pLPG:pWCAG) resulted in more than a 10-fold titer drop. Interestingly, a 2-fold decrease in the number of packer allowed increasing titer more than 4-fold relative to the standard. We used this option in subsequent experiments for obtaining viral preparations.

1. Titers of virus preparation used for chicken cells transformation in vivo depending on the quantitative ratio between the components of the vector system used to obtain it

Virus titer	1:2:3	(1:2:3)/2	1:4:3	1:4:6	2:2:3	1:1:3
Physical, number of RNA copies/ml	5.6×10^9	2.9×10^9	6.9×10^8	5.1×10^8	5.2×10^9	1.9×10^{10}
Biological, CFU/ml	6.7×10^6	3.4×10^6	8.3×10^5	5.8×10^5	6.3×10^6	2.4×10^7

N o t e. Quantitative plasmid ratio (psPAX2, pLPG, and pWCAG, respectively).

As a result, we obtained titers of 2.4×10^7 CFU/ml for the vector with CAG-promoter. Biological virus titer was determined in 293T cells by eGFP fluorescence. The amount of virus in the preparation was evaluated by specific RNA content in RT-PCR (Bio-Rad, USA). The vector titer was 6.2×10^8 CFU/ml after concentration by ultracentrifugation.

2. Evaluation of the efficiency of the pWCAG lentiviral vector in vivo introduction in embryonic cells of the Ptichnoe cross chickens depending on embryo age

Group (incubation time, hour	Sample number	Number of vector copies per diploid cell genome	Proportion of embryonic cells expressing eGFP,%
Group I (20-24)	1-1	0.96	
	1-2	1.86	
	1-3	0.33	
	1-4	0.10	
	1-5	0.36	
	1-6	0.14	
	1-7	5.52*	
	1-8	0.68	
	1-9	0.44	
	1-10	2.20	
mean efficacy		0.78 (78 %)	
	1-11		44.8
	1-12		33.1
	1-13		28.4
	1-14		32.8
	1-15		32.5
mean efficacy			34.3
Group II (50-55)	2-4	0.30	
	2-5	0.30	
	2-6	0.38	
	2-7	0.00	
	2-8	0.70	
	2-9	0.34	
	2-10	0.15	
mean efficacy		0.31 (31 %)	
	2-11		15.9
	2-12		42.7
	2-13		13.7
	2-14		37.8
	2-15		39.7
mean efficacy			30.0

N o t e. In determining average efficiency, samples marked with an asterisk (*), were not taken into account.

The resulting virus preparation was used for introduction into embryonic cells at different times:, i.e. chicken embryos of group I were infected after 20-24 hours of incubation, and in embryos of group II it was after 50-55 hours of incubation (Table 2). At incubation day 7, eggs were opened, embryos tissue fragments were collected as the samples for chromosome DNA extraction and analyze of eGFP expression. DNA samples with the value of $OE_{260/280}$ of not less than 2 were the benchmark for normalization of other samples. All calculations were made based on the fact that the molecular weight of diploid *Gallus gallus* genome was 2.5 pg.

According to RT-PCR results, the average efficiency of lentiviral vector introduction in chick embryo cells in group I was 0.78 copies per diploid cell genome, i.e. 78.0 % of embryonic cells contained an integrated copy of DNA

vector. In group II the value was 2.5 times lower (31.0 %). At the same time, there were 0.10 to 5.52 copies per cell genome in embryo group I, and in four of 10 samples this value was at least one proviral copy per diploid genome, while in group II the range was much lower making from 0.00 to 0.70 proviral DNA copies. The study of marker *eGFP* gene expression in embryonic tissues using flow cytometry revealed it in 34.3 % of embryonic cells on average in group I and in 30.0 % of embryonic cells in group II. Thus, in group II, indicators of the introduction efficiency for the vectors produced using RT-PCR and flow cytometry were almost identical (31.0 and 30.0 %, respectively), while in group I they differed 2.3 times (78.0 and 34.3 %, respectively; see Table 2).

Based on the results, the average efficiency of gene transfer using lentiviral vectors was 30.0-34.3 %, slightly varying with the changes in the time of introduction after the beginning of embryo incubation. Probably, only part of embryonic cells was available for virus infection. Since viral preparations with similar titers were used with both incubation periods, the observed difference in the number of vector copies in cell genome in embryo groups I and II was probably due to the fact that more viral particles penetrated embryo cells at earlier stages (see Table 2). This suggests that chicken embryos can be infected with lentiviral preparations with predicted efficiency via varying the viral preparation titres and the time of introduction after the beginning of embryo incubation, thereby producing transgenic chickens with the expected gene «dose» (number of copies) according to the experimental purpose. Thus, the efficiency of chicken embryo thransgenesis using lentiviral vectors does not depend on the stage of embryonic development at least for 55 first hours of incubation and can be predictable.

Thus, the study of the conditions of lentiviral vector introduction in chicken embryonic cells showed that biological titers of viral preparations of about 10^8 CFU/ml were sufficient to infect up to 78 % of the cells in the early stages of embryo development. Changing in the ratios of the vector system components compared to the standard scheme allows to significantly increase the titers of the virus preparations produced. The selected conditions may be used as a tool for the study of the factors influencing the level and stability of transgene expression in chicken cells in vivo in future experiments.

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