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Genome and Its Regulation

Study of transient expression of human *apo*A-1 gene under control of various promoters in CHO-K1 cells

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The recombinant plasmids containing the genomic human apoA-1 gene under the transcriptional control of hybrid promoters, namely, the cytomegalovirus immediate early enhancer/chicken ?-actin promoter and first intron (CAG promoter) and cytomegalovirus immediate early enhancer/promoter with intron A (hCMVintronA promoter) have been constructed. The transient expression of human apoA-1 gene has been studied in CHO-K1 cells using designed and pTRapo vectors. The expression of the transgene for transfected with pTRapo and pTRapochCMVintronA CHO-K1 cells have been shown.

Key words: *transient expression, apolipoprotein A-1, CHO-K1.*

Introduction. The way of exogenous DNA delivery into mammalian cells is extremely important to study gene functions as well as to advance gene therapy. At present there are various approaches to the introduction of a heterologous DNA into mammalian cells, among them the usage of vectors, based on mammalian viruses, physical (electroporation in particular) and chemical (usage of liposomes, polycations etc) methods etc. Each of these approaches is aimed at solving definite practical tasks.

Vectors, designed on the basis of viruses, are widely exploited in gene therapy due to their high specificity and effectiveness of gene transferring *in vivo* [1]. However, the usage of virus vectors in gene therapy is related to such problems as toxicity, increased immunogenicity, and ability to activate protooncogenes and induce the formation of pathogenic variants of viruses due to recombination with endogenous viruses. Therefore, the elaboration of non-virus systems of genetic material introduction is a perspective direction in this area [2].

The plasmid vectors, which may be applied at cells transfection by physical or chemical methods, do not have disadvantages, inherent to virus vectors, though they are characterized by much lower effectiveness of transfection *in vivo* in comparison with the latter at their usage for genes transfection *in vivo* [3]. On the other hand, the easiness of obtaining and manipulation with recombinant plasmids made them one of the main vectors for genes transfer to obtain stable transformants and transient cultures which may be used while producing recombinant proteins with therapeutic properties in mammalian cells *in vitro* [4].

It was shown that the expression level of genes, introduced into mammalian cells, depends on many reasons, including the strength of transcription regulatory elements [5]. To correct disorder, caused by the deficiency of expression of a specific gene in the organism, high level of expression of a target transgene is often necessary to achieve physiological concentrations of the protein and therapeutic effect correspondingly. One of the perspective strategies of solving the problem, connected with a low level of transgenes expression, is optimization of already known regulatory sequences and search for stronger ones. The advantage of using strong regulatory elements is the possibility to decrease the amount of the vector, which is introduced *in vivo* to achieve therapeutic effect, or, in case of recombinant proteins production, to increase their production. To some degree it might solve the problem of low effectiveness of cells transfection by the plasmid vectors or decrease possible negative consequences at the usage of vectors, designed on the basis of viruses.

The purpose of this paper was the construction of expression vectors, containing genome variant of human apolipoprotein A-1 (apoA-1) gene under the transcriptional control of different hybrid elements, as well as the study of transient expression of human apoA-1 gene in CHO-K1 cells.

Materials and methods. *Plasmids and bacterial strains*. Genome variant of human *apoA-1* gene was used as a transgene, the source of it was recombinant plasmid *pTRapo* [6].

The expression vectors were designed using plasmid *pTR-EGFP* [6], *pBacMam-1* (Novagen, USA), *pTR-GT60* [7] and *pTRUF*, kindly presented by Dr. S. Zolotukhin (The Centre of Gene Therapy, USA), *pBluSKM* (Fermentas, Lithuania) and bacterial strain *Escherichia coli* DH10B *(FÏmcrA (mrrÏhsdRMS-mcrBC) ц80dlacZ M 15 lacX74 deoR recA1 endA1 araD139 (ara, leu)7697 galU galK lÏrpsL nupG)* (Invitrogen, USA).

The work with bacterial cultures, the transformation of *E.coli* cells, the restriction and ligation of DNA was performed by standard methods as described [8]. The obtained recombinant plasmids were checked by the restriction analysis.

Isolation and purification of plasmid DNA. Plasmid DNA was isolated by the method of alkaline lysis [8]. Additional purification of plasmid from RNA and proteins admixtures was conducted by treating the preparation with ribonuclease A (USB, USA), by the method of phenol-chloroform extraction [8] and PEG 8000 precipitation [9]. DNA concentration and purity were defined by spectrophotometry according to the ratio A_{260}/A_{280} [8] on the Specord UV VIS device (CARL ZELSS JENA, Germany) as well as by electrophoresis method in 0.8% agarose gel.

Cells cultures. The cell line of Chinese hamster CHO-K1, obtained from the Russian collection of cell cultures (St. Petersburg) was used in the work. The cells were cultivated at 37°C in the atmosphere, containing 5% CO_2, in the F10 medium (Sigma, USA) with the addition of 10% embryonic calf serum (Genom, Ukraine), 100 OD/ml penicillin and 100 mg/ml streptomycin (Kyivmedpreparat, Ukraine).

Cells transfection. Two days before transfection the cells were sown in the quantity of $8C10^5$ on Petri dishes, 6cm diameter. The transfection was conducted using branched polyethyleneimine (PEI) (25 kDa, Aldrich, USA) by the method, described in the work [10]. DNA/PEI preparation was prepared in mass ratio 1:2. For transfection 3.5мg of plasmid DNA was applied to each dish. The time of cells contact with transforming mixture was 1 hour.

The transfection effectiveness was defined using the transfer of *pTR-EGFP* vector into CHO-K1 cells, which contains a marker gene of green fluorescent protein (egfp) [6].

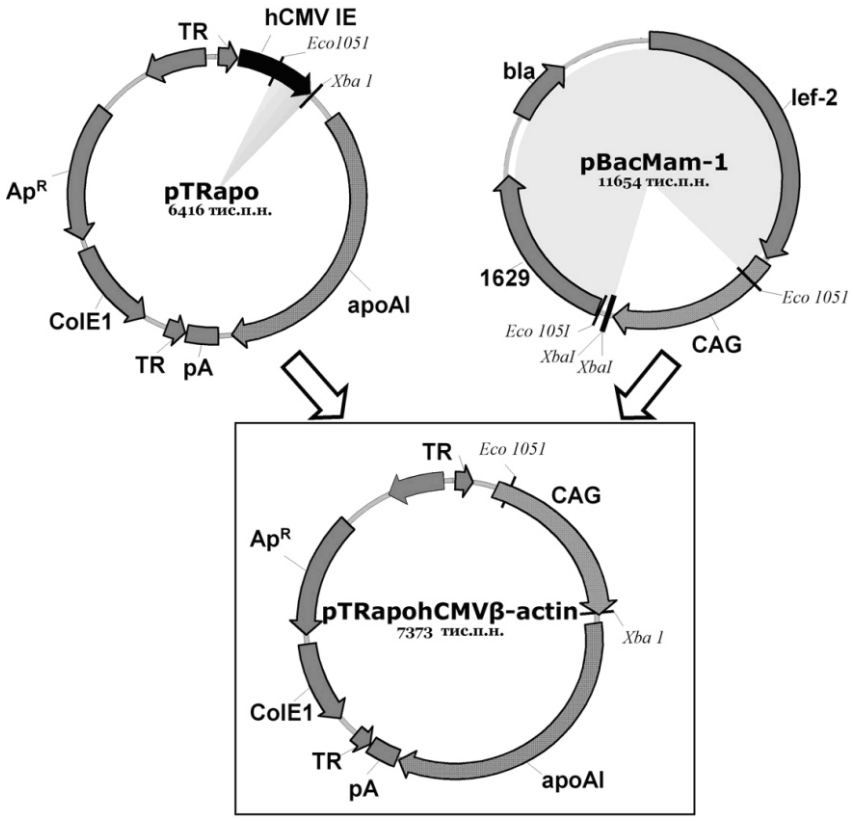

Figure 1. The scheme of obtaining the recombinant construction *pTRapohCMVe-actin*: apoA-1 – human gene of apolipoprotein A-1; hCMV IE – cytomegalovirus immediate early gene enhancer/promoter; CAG – human cytomegalovirus immediate early gne enhancer, chicken в-actin gene promoter, chicken first intron в-actin gene; ITR – inverted terminal repeats of adenoassociated virus; pA – polyadenilation site

The analysis of apoA-1 synthesis. The level of human apoA-1 accumulation in a cultural medium was determined by the methods of Western-blot and dot-blot-analysis. The proteins of cultural medium were concentrated previously using trichloroacetic acid [11], the precipitate was separated from the supernatant by centrifugation, and dissolved in the buffer, containing 4M urine and 0.1M Tris. The samples for proteins separation in Ds-Na-PAAG were prepared according to the method [8]. The proteins were separated in 12% Ds-Na-PAAG. For immunodetection the proteins from gel were transferred to PVDF membrane (Amersham Biosciences, Sweden). Free sites on membrane were blocked by 3% solution of non-fat dry milk. The membrane was incubated for 1 hour with monoclonal antibodies against human apoA-1 (MGHLaa 1/500, IMTEK, Russia). In the following experiment the membrane was incubated in the course of 1 hour with antibodies against mouse G immunoglobulins, conjugated with horse radish peroxidase (P-GAM 1/1000, IMTEK). Immune complexes were visualized using the ECL method [12], and luminol (Sigma) as a substrate.

Chemiluminescence was registered using the X-ray film Retina XBM (Lizophorm, Ukraine). The obtained images were scanned and the quantitative estimation of signals intensity was performed using Total Lab program (Amersham Biosciences). Human apoA-1 protein (Sigma) was a standard of molecular weight and concentration.

The ApoA-1 protein content in cultural medium was also determined by the method of indirect immune-enzyme analysis (ELISA).

The following reagents were used for immunodetection: monoclonal antibodies against human apoA-1 (MGHLaa 1/2700, IMTEK) and antibodies against mouse G immunoglobulins, conjugated with horse radish peroxidase (P-GAM 1/5000, IMTEK). Vacant sorption sites were blocked by 0.01M phosphate-saline buffer (PSB), containing 0.1% twin-20. TMB (Sigma) was used as a substrate; the extinction was measured at the wave length of 450nm on many-channel photometer Multiscan MCC/340 (Titertek, USA).

Results and discussion. The promoter of immediate early gene for human cytomegalovirus - hCMV-IE is most frequently used as an element of transcription regulation of heterologous genes in mammalian cells. First of all, it is conditioned by such characteristics as high transcription rate, absence of

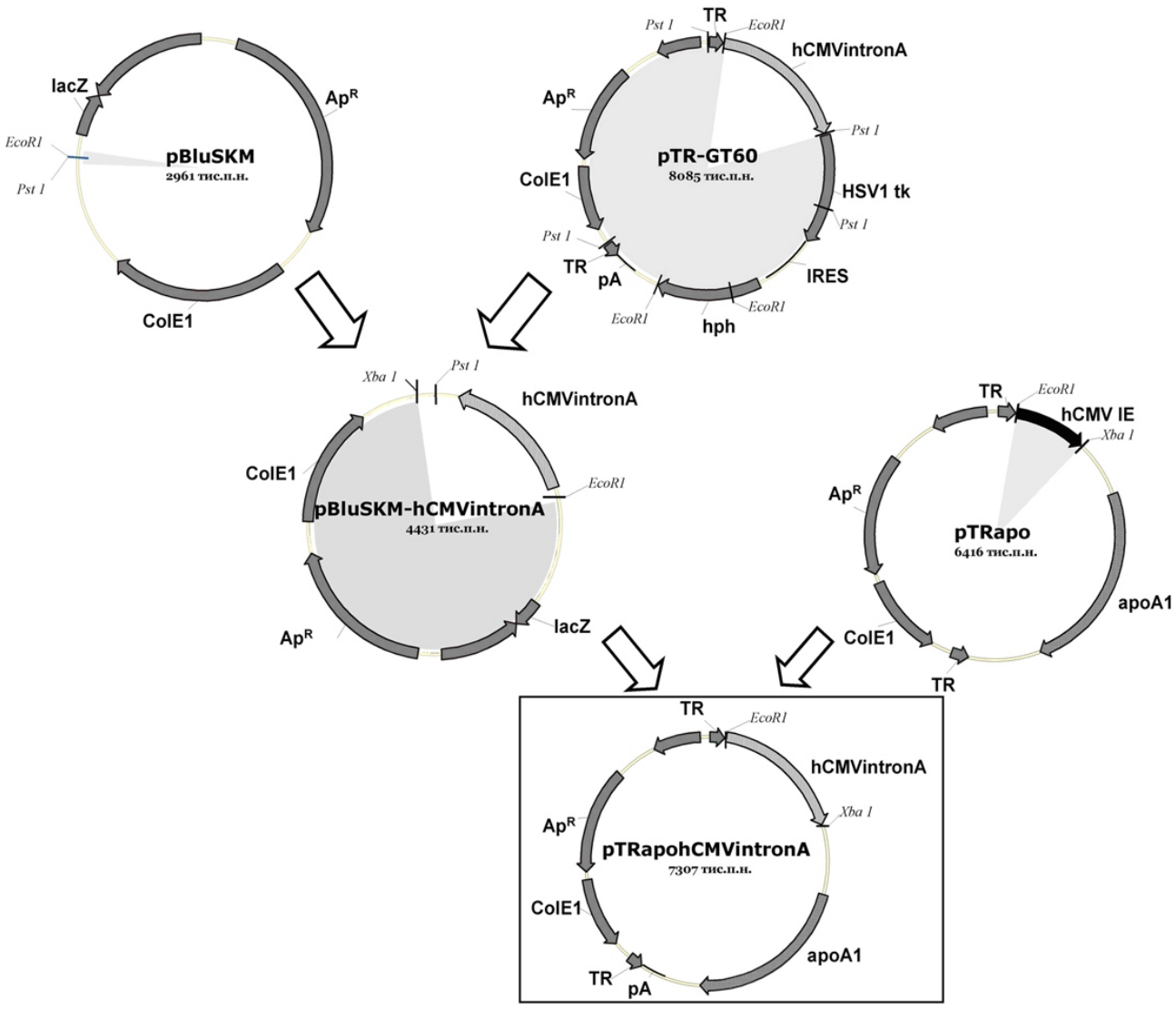

Figure 2. The scheme of obtaining the recombinant construction pTRapohCMVintronA: apoA-1 – human gene of apolipoprotein A-1; hCMV IE – cytomegalovirus immediate early gene enhancer/promoter; hCMVintronA - human cytomegalovirus immediate early gene enhancer and human cytomegalovirus immediate early gene enhancer with intron A; ITR – inverted terminal repeats of adenoassociated virus; pA – polyadenilation site

tissue specificity and, at the same time, size compactness (700 n.p.) [13, 14].

It was shown that the including of some genome elements, introns and 3'- and 5'-non-translated sequences, in particular, into cDNA of target genes increases the level of transgene expression both *in vitro* and in transgene animals [15]. The mechanism of such sequences influence on the level of transgenes expression is not defined in all the cases, but it is thought to be connected with the phenomena of increasing transcription rate, the rise of stability, transport and/or the effectiveness of 3'-end mRNA formation at its maturing [16].

At present a large quantity of hybrid elements of the transcription regulation is created [16-18]. It was determined in the previous works [5, 19] using marker genes, that hybrid sequences – human cytomegalovirus immediate early enhancer/chicken

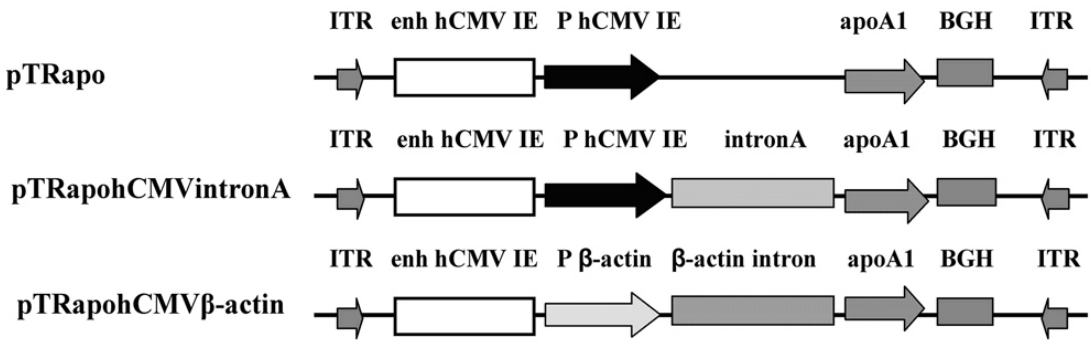

Figure 3. The general scheme of regulatory elements location: ITR – inverted terminal repeats of adenoassociated virus; pA – polyadenilation site; enh hCMV IE - human cytomegalovirus immediate gene early enhancer; P hCMV IE - human cytomegalovirus immediate early promoter; intron A - human cytomegalovirus immediate early gene enhancer with intron A; P в-actin - chicken в-actin gene promoter; в-actin intron - first intron of chicken в-actin gene; BGH pA – polyadenilation site of ox hormone growth gene; apoA-1 – human gene of apolipoprotein A-1

в-actin promoter with chicken first intron of в-actin gene (CAG sequence) and enhancer/promoter of cytomegalovirus immediate early gene with intron A of the same gene (hCMV-intronA sequence) provide the highest level of transgene expression *in vitro* and *in vivo*. Therefore, the usage of these regulatory elements for the increase of expression level of a target gene in mammalian cells is perspective.

To estimate the influence of abovementioned hybrid promoters on the expression of human *apoA-1* gene *in vitro* we designed several plasmid vectors.

While designing the expression vector *pTRapo-hCMVв-actin* in *pTRapo* plasmid on sites of restrictase *Eco1051-XbaI* there was a substitution of hCMV-IE promoter for hybrid CAG sequence (Fig.1).

The *pTRapohCMVintronA* plasmid was designed through the intermediate construction *pBluSKM-hCMVintronA.* To obtain this plasmid the hybrid sequence hCMVintronA was cloned on the sites of restrictase *EcoRI-PstI* from the plasmid *pTR-GT60* into a *pBluSKM* vector, which was hydrolized by restriction enzymes *Eco-RI-PstI*. Then promoter hCMV IE was substituted in plasmid *pTRapo* on the sites of *EcoRI-XbaI* restriction for hybrid sequence hCMVintronA (Fig.2).

In the investigated vectors human apoA-1 gene was located under the control of different hybrid sequences: hCMVintronA, CAG and hCMV IE promoter. These elements have a common enhancer – human cytomegalovirus immediate early gene enhancer and different promoters: human cytomegalovirus immediate early promoter in case of hCMV IE and sequence hCMVintronA, and chicken в-actin promoter for CAG element. Besides, hybrid sequences hCMVintronA and CAG include first introns: of human cytomegalovirus immediate early gene and chicken в-actin gene (Fig.3).

The peculiarities of transgene expression under the control of mentioned elements were studied *in vitro* at transient synthesis of apoA-1 in CHO-K1 cells. The advantages of using transient transformers for such an analysis is the fact that the expression level does not depend on the “position effect” i.e. regulatory chromosome elements do not influence it, as transgene expression occurs from non-integrated vectors. Moreover, much less time is needed to conduct such investigations than to obtain and analyze stable transformants [18].

It is worth mentioning that human apoA-1 protein is secretory. ApoA-1 gene contains a sequence, coding its own signal peptide, which provides its secretion into the medium. In this regard apoA-1 was detected directly in the cultural medium according to the following scheme: in 24 hours after the transfection the medium was changed for non-serum one, next day it was collected completely and frozen, while the fresh one was added to the cells. Thus, the samples of cultural medium were taken on the 2^{nd}-5^{th} days after the cells transfection.

On the second day after transfection the presence of target protein was detected by the method of immune-chemical analysis in the cultural medium of cells, transfected by the expression vectors *pTRapo* and *pTR-apohCMVintronA*.

The example of experiment is presented at Fig.4. The ELISA testing of cultural media using did not give positive results (the data are not shown), which is connected with insufficient sensitivity of the method for the detection of the quantity of apoA-1 protein, which

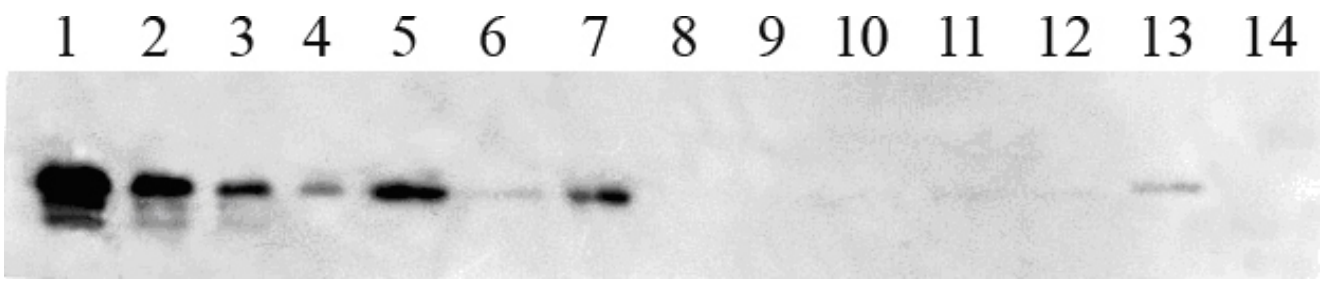

Figure 4. The Western-blot analysis of secretion of apoA-1 protein into the cultural medium by CHO-K1 cells, transfected by different expression vectors: 5-7 – by *pTRapohCMVintronA* expression vector; 8-10 – *pTRapohCMVe-actin*; 11-13 – *pTRapo*; 14 – control, cultural medium, containing non-transfected CHO-K1 cells. The sample, introduced into the socket corresponds to 280мl of cultural medium; 1-4 – standard of molecular weight and concentration of human apoA-1 protein (Sigma, USA) (1-5 ng, 2 – 1.67 ng, 3 – 0.55 ng; 4 – 0.18 ng)

was synthesized by CHO-K1 cells. The comparison of plasmids with different elements of transcription regulation showed that *pTRapo-hCMVintronA* construction provides a higher level of transgene expression than *pTRapo* plasmid.

The immunodetection showed that the level of apoA-1 synthesis by cells, transfected by *pTRapohCMVintronA* plasmid, amounts to about ~3nm of protein per 1ml of medium a day, while at cells transfection by *pTRapo* palsmid it is ~0.4nm/ml. Not high level of apoA-1 synthesis at the conditions of this experiment may be explained by insufficient effectiveness of cells transfection by a gene vector.

The dynamics analysis of protein accumulation in the samples of cultural medium using dot-blotting did not reveal apoA-1 protein starting from the 3rd day. The transgene expression from plasmid vectors *in vitro* during a week was described for transient cultures, longer expression is impossible due to the loss of a transgene (at the elimination of plasmid DNA, division or death of cells).

The level of transgene expression, controlled by hybrid CAG sequence, was lower than the sensitivity of the used analysis method (<0.2 ng/ml). The designed genetic constructions will be further employed for the obtaining and analysis of human apoA-1 gene expression by stable transformants of CHO-K1.

Conclusions. The recombinant plasmids were designed, containing genome variant of human qpoA-1 gene under the transcriptional control of different regulatory elements. The expression of human apoA-1 gene on the level of ~0.4 and ~3 ng of protein per 1ml of the medium was shown for CHO-K1 cells, transfected correspondingly by *pTRapo* and *pTRapohCMVintronA* plasmids on the 2nd day after the transfection. The obtained data on the increase in human apoA-1 gene expression under the control of hybrid *hCMVintronA* sequence in comparison with the level of transgene expression under the hCMV IE promoter in general are in good agreement with the results of other researches.

Ю. Н. Гильчук, Е. М. Сухорада, Т. А. Рубан, Е. К. Топорова, В. А. Кордюм

Изучение транзиторной экспрессии гена *апоА-1*человека под регуляцией различных гибридных последовательностей в клетках CHO-K1

Резюме

Сконструированы рекомбинантные плазмиды, содержащие геномный вариант гена аполипопротеина А-1 (апоА-1) человека под транскрипционным контролем гибридных последовательностей: энхансер среднераннего гена цитомегаловируса человека/промотор гена -актина цыпленка с первым интроном гена -актина цыпленка (CAG последовательность) и энхансер/промотор среднераннего гена цитомегаловируса человека с интроном А того же гена (hCMVintronA последовательность). С использованием полученных векторов и плазмиды pTRapo изучена транзиторная экспрессия гена апоА-1 человека в культуре клеток CHO-K1. Для клеток, трансфицированных векторами pTRapo и pTRapohCMVintronA, показана экспрессия трансгена.

Ключевые слова: транзиторная экспрессия, аполипопротеин А-1, CHO-K1.

REFERENCES

1. *Romano G., Micheli P., Pacilio C., Giordano A.*Latest developments in gene transfer technology: achievements, perspectives and controversies over therapeutic applications // Stem cells.–2000.–**18**.–P. 19–39.

2. *Богданенко Е. В., Свиридов Ю. В., Московцев А. А., Жданов Р. И.* Невирусный перенос генов *in vivo* в генной терапии // Вопр. мед. химии.–2003.–№ 3.–С. 226–245.

3. *Nishikawa M., Hashida M.* Nonviral approaches satisfying various requirements for effective *in vivo* gene therapy // Biol. Pharm. Bull.–2002.–**25**.–P. 275–283.

4. *Florian M.* Wurm production of recombinant protein therapeutics in cultivated mammalian cells // Nat. Biotechnol.–2004.–**22**.–P. 1393–1398.

5. *Xia W., Bringmann P., McClary J., Jones P. P., Manzana W., Zhu Y., Wang S., Liu Y., Harvey S., Madlansacay M. R., McLean K., Rosser M. P., MacRobbie J., Olsen C. L., Ronald R.* Cobb
high levels of protein expression using different mammalian CMV promoters in several cell lines // Protein Exp. and Purif.–2006.–**45**.–P. 115–124.

6. *Топорова Е. К., Новикова С. Н., Лихачева Л. И., Сухорада, Рубан Т. А., Козел Ю. Н., Иродов Д. М*. Невирусная доставка гена *апоА-1* человека в клетки млекопитающих *in vitro* и *in vivo* // Біополімери і клітина.–2004.–№ 1–2.–С. 25–32.

7. *Кордюм В. А., Топорова Е. К., Окунев О. В., Похоленко Я. А., Сухорада Е. М., Рубан Т. А., Андриенко В. И., Иродов Д. М.* Новая множественно маркированная линия клеток–производная от CHO-K1 // Біополімери і клітина.–2003.–**19**, № 3.–С. 252–256.

8. *Sambrook J., Fritsch E. F., Maniatis T*. Molecular cloning: a laboratory manual.–New York: Cold Spring Harbor Lab. press, 1989.–Vol. 1.

9. *Frederick M. Ausubel F. M., Brent R., Kingston R. E., Moore D. D., Seidman J. G., Smith J. A., Struhl K*. Current protocols in molecular biology.–New York: John Wiley and Sons, 1997.–Vol. 1.

10. *Boussif O., Lezoualch F., Zanta M. A., Mergny M. D., Scherman D., Demeneix B., Benr J.-P.* A versatile vector for gene and oligonucleotide transfer into cells in culture and *in*
vivo: polyethylenimine // Proc. Nat. Acad. Sci. USA.–1995.–**92**.–P. 7297–7301.

11. *Bollag D. M., Rozychi M. D*. Protein Methods.–New York: Wiley-Liss. Inc., 1996.–415 p.

12. *The Protein* Protocols Handbook / Ed. J. M. Walker.–Totowa, New Jersey: Humana press Inc., 2002.–1139 p.

13. *Boshart M., Weber F., Jahn G., Dorsch-Hasch K., Fleckenstein B., Schaffner W.* A very srong enchancer is located upstream of an immediate early gene of human cytomeralovirus // Cell.–1985.–**41**.–P. 521–530.

14. *Fitzsimons H. L., Blaund R. J., During M. J.* Promoter and regulatory elements that improve adeno-associated virus transgene expression in the brain // Methods.–2002.–**28**.–P. 227–236.

15. *Palmiter R. D., Sangren E. P., Avarbock M. R., Allen D. D., Brinster R. L*. Heterologous introns can enhance expression of transgenes in mice // Proc. Nat. Acad. Sci. USA.–1991.–**88**.–P. 478–482.

16. *Chapman B. S., Thayer R. M., Vincent K. A., Haigwood N. L*. Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells // Nucl. Acids Res.–1991.–**19**.–P. 3979–3986.

17. *Niwa H., Yamamura K., Miyazaki J*. Efficient selection for high-expression transfectants with a novel eukaryotic vector // Gene.–1991.–**108**.–P. 193–199.

18. *Savvas C.* Makrides components of vectors for gene transfer and expression in mammalian cells // Protein Exp. and Purif.–1999.–**17**.–P. 183–202.

19. *Xu Z.-L., Mizuguchi H., Ishii-Watabe A., Uchida E., Mayumi T., Hayakawa T.* Optimization of transcriptional regulatory elements for constructing plasmid vectors // Gene.–2001.–**272**.–P. 149–156.

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