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GENE THERAPY OF MASS PATHOLOGIES

The whole complex of investigations is described that allows to run the way from the idea to experimental realization of a fundamental possibility of gene therapy and its application to mass pathologies on the sample of insulin-dependent diabetes and atherosclerosis.

The search and analysis were performed of such regulatory elements that whould permit an expression irrespective of the state of a general cell regulation.

Expression of the implanted gene material is shown on cells of different tissues and different organisms and also in vivo (both the model gene of β-galactosidase Escherichia coli and insulin- and apolipoprotein high density A1-coding genes related to above stated pathologies). It is concluded that the results obtained in the culture outside the organisms shouldn't be extrapolated on the animals, i. e. on the organism's level.

The expression of recombinant molecules is shown to be ambiguous and depend on gene's surroundings and the type of recipient cells. Both in the culture and in the organism the individual cell heterogeneity is observed in the quantitative characteristics of the expression of gene implanted from outside. Besides, an individual organism and age heterogeneity with regard to the expression of the exogenous gene is reported.

The conclusion is made about the necessity to individualize gene therapy of mass pathologies.

Introduction into the problem. Gene therapy has passed for almost 20 years of its existence an exceedingly bright and sometimes dramatic way. However now when it has achieved a level of the practical realization its pioneering significance for the medicine is evident. But medicine on the whole being on its level and biomedicine as its advanced trend in particular are far more ahead with qualitatively new problems in store. Now we witness practical development of the human biotechnology, i. e. the technology of the direct transformation of human whose aim is a biological reconstruction of a man as an individual. Terms (direct biotechnology of a man) and the aim (biological reconstruction of a man) are still new and unusual. Just as the term «gene engineering of human» (followed by the term «gene therapy») and especially tasks and potentialities of the technology behind the terms caused professional contradictions of specialists and shocked the public new problems may also first cause indignation. Nevertheless the experience of the human civilization shows that nothing can stop the new in science. One may surely predict that biotechnological transformation of human with all its methods and potentialities will soon become habitual.

Direct biotechnology of a man embraces the technology of cell implantation, reproduction technologies, technologies of artificial organs and tissues, etc. But gene therapy is most radical trend that essentially interacts with all the rest and unites all in one. It has started from theoretical and experimental substantiation of treatment of classical monogenic hereditary diseases gradually expanding its activities to all the processes proceeding in human organism. Previously we formulated a concept of gene therapy of mass pathologies [1, 2]. So almost all human

diseases are in the sphere of potentialities of gene therapy. However mass pathologies require a specific approach whose peculiarities are not only pure methodical but also fundamental.

The first (and conceptually the main) peculiarity is a fundamental difference in monogene hereditary diseases and mass pathologies. In the first case there is a particular defect in a particular gene. So, the whole problem (no matter how complex it would be) is reduced to elimination of this defect (either by alteration or by complementation at the expense of introduction of a sound gene under the same outside regulation). In a case of mass pathologies genes themselves are not distorted. Something as a rule unknown at the level of primary processes is happening with the regulation system which disturbs functioning of these or other systems of the organism. Though, finally, it affects functioning of particular genes but the genes themselves either unchanged or changed in few cells (for example tumor ones) or these changes are not functionally essential.

So, in most cases a simple introduction of a gene will have no effect. One should create such molecular construction where the introduced gene will function despite of the changed regulation that makes the same sound organism's own gene available in a cell function poorly.

The second difference (also conceptual) is as follows. In case of classical monogenic diseases the damage is available only in one gene. The processes in the organism are controlled by corresponding metabolic chains whose activity is specified by gene ensembles. All genes in each of these ensembles act normally except one particular gene. It is enough to alter one particular gene (or to complement its activity) and the metabolic chain will become sound.

It is assumed for mass pathologies that the whole metabolic ensemble, a metabolic chain and often many metabolic chains are in a disbalance. So, the question is here what one should do in this situation since it is impossible to complement all genes.

However the analysis of metabolism shows the following. Metabolism is organized so that there is a small number of key controlling processes able to a considerable extent to eliminate or to smooth damage of the rest links of the chain. So the concept of gene therapy of mass pathologies is underlain by an idea of key arresting processes and, respectively, the genes coding them. But this concept must be experimentally confirmed.

Finally, in case of hereditary diseases the gene is defect from birth and even earlier — from gamete and will never after by itself. So it must be altered (complemented) for the rest of life. In most cases of mass pathologies the gene capable to stop pathology (as the rest of the genes from the damaged ensemble) is sound. It needs no alteration but only complementing for the period until organism normalizes its processes. Though disfunction of the gene in this case is not prolonged still the organism needs time to normalize its processes and this time will vary in different cases. For young it will be short (all processes in this period normalize rather quickly). For middle age (and even for young but with chronic disease when the organism is exhausted) the time for normalization is increased. But with gene therapy of age pathologies and diseases specified by the death of nonrestored cells the presence of the introduced gene must be permanent. So, if for classical gene therapy the introduction system must provide a permanent stay and functioning of the introduced gene material then in a case of mass pathologies it is necessary to have all variations of the stay of recombinant molecules in the organism cell — from short-term to permanent. It needs additional methodical designs.

As a result of the performed complex studies we have succeeded to run the way from the idea to experimental realization of a fundamental possibility of gene therapy of mass pathologies. This work describes this way.

Materials and methods. Strains, media. Strains *Escherichia coli* (HB101, DH1, JM101, LE392) and yeasts *Saccharomyces cerevisiae* LL20 used in the work were obtained from the collection of cultures at the Institute of Molecular Biology and Genetics of the Academy of Sciences of Ukraine. A library of the human genes were received from N. S. Neznanova (Institute of Molecular Genetics of the Russian Academy of Sciences, Moscow).

Standard methods for growing of microorganisms and cultivation of somatic cells on corresponding media are used in the work [3, 4].

Cells of embryonal human lung LEH, diploid fibroblasts of human skin SL, cultivated mouse cells of C3H10TI/2 and C3HLtk⁻ lines and

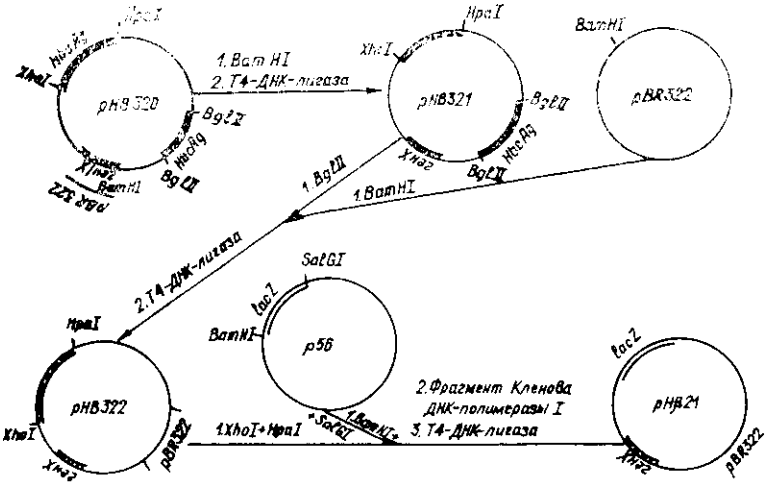


Fig. 1. Construction scheme of plasmid *pHβ21* carrying gene of bacterial β-galactosidase under control of the promoter of the surface antigen in hepatitis B virus

cultivated cells of Chinese hamster of Bild-ii-FAF28 line of clone 237-8Glu⁻ts were the object of investigation. LEH and C3HLtk⁻ cells were obtained from the collection of the Institute of Virology of the Academy of Medical Sciences of Russia, cells C3H10TI/2 and 237-8Glu⁻ts — from the Institute of Molecular Genetics of the Academy of Sciences of Russia, cells SL — from the Michigan State University, USA.

Plasmids. Three initial plasmids for producing the rest of recombinant DNA were used in the work. These are *pUC18* from the Institute of Molecular Biology and Genetics of the Academy of Sciences of Ukraine, a standard commercial vector for cloning of DNA, *pAL1*, a derivative from *pUC18* containing one human *alu*-repetition by *Bam*HI-site of a polylinker presented by N. V. Tomilin (Institute of Cytology of the Academy of Sciences of Russia, S. Petersburg), *pYF92* [5] used for cloning of ARS-sequences of DNA presented by V. L. Larionov (Institute of Cytology of the Academy of Sciences of Russia, S. Petersburg).

DNA of plasmid *pGA293* kindly presented by Dr. Siminovich (Canada) and *pCH110* containing *lacZ* gene of *E. coli* that codes a synthesis of bacterial β-galactosidase were used in the experiments on study of bacterial β-galactosidase expression [6]. Construction of *pAG293A* is a plasmid *pGA293* with *alu*-repetition cloned by *BamHI*-site while plasmid *pGA293Z*$^{\Delta}$ is devoid of *EcoRI*-fragment containing *lacZ* gene.

Besides we used also plasmids *pLZ56* and *pHB320*, *pHβ21*. Plasmid *pLZ56* was obtained from V. Korobko (Shemyakin Institute of Biological Chemistry of the Russian Academy of Sciences, Moscow). It contains *lacZ* gene of *E. coli* and was used in the control of tandem of earlier promoters A_2 and A_3 of phage T7. Plasmid *pHB320* was obtained from Dr. E. Gren (Institute of Organic Synthesis of the Latvian Academy of Sciences, Riga). It is *pBR322* with a full DNA replica of hepatitis virus

B. This plasmid was used to construct *pHβ21*, its construction scheme is presented in fig. 1.

Gene of human insulin containing a promoter but without a regulatory site providing tissue-specificity of the expression (*BglII-TaqI*-fragment) [42] was cloned in the bacterial plasmid *pBR322ins* and *pAins* (the latter contains *alu*-repetition).

Besides in a number of experiments we used a recombinant construction on the base of hepatotropic vector *pGins* and restriction fragment of DNA containing insulin gene (ins).

Screening of the human genes library, manipulation with plasmid DNA and obtaining recombinant molecules were realized by the conventional procedure described by Maniatis [7]. Fourty-member oligonucleotide synthesized at the Institute of Molecular Biology and Genetics of

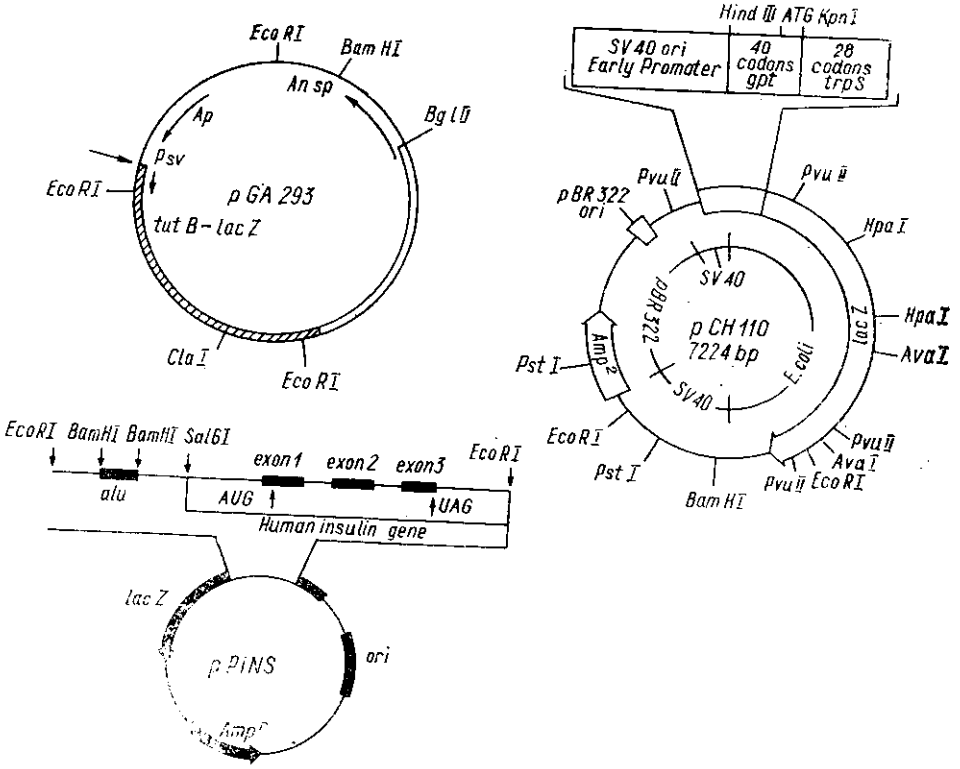


Fig. 2. Samples of some plasmid constructions used in experiments

the Academy of Sciences of Ukraine and complementary to the structural part of the human gene was used as a probe. Constructions of the main used plasmids are presented in tabl. 1 and fig. 2. Plasmid DNA was isolated from the yeast cells as recommended by Glover [4]. Plant DNA was isolated as described in [8].

Primary DNA structure was determined by the Maxam — Gilbert method [9].

Enzymes. Restriction endonucleases and the Klenov fragment of DNA-polymerase I were received from the Scientific-Production Association «Ferment» of the Research Institute of Applied Enzymology (Vilnius). DNA-ligase of phage T4 was kindly given by B. Troyanovsky and Yu. Gorlov (Institute of Molecular Biology and Genetics of the Academy of Sciences of Ukraine).

Liposomes. Negatively-charged monolamellar liposomes from 10 to 100 nm in size were used as DNA carrier. They consists of (%): lecitin — 70, cholesterin — 20, dicethylphosphate — 9, phosphatydylamine — 1. Concentration of lipids was 20 mg/ml. Plasmid DNA was enclosed into the liposomes by the method of Ca-fusion. The quantity of DNA enclosed

into the liposomes was 7—10 % of the introduction amount. Sizes, lamellation and preservation of liposomes were controlled by means of electron microscopy of slices [10].

Production and purification of immune serums. Chinchilla rabbits were vaccinated with purified β-galactosidase isolated from *E. coli*. Injections were made into cervical and popliteal limph nodes. For the first vaccination we used a complete Freund adjuvant, for the second and third ones — an incomplete adjuvant. Buster injections were performed intravenously. Each animal was injected with 0.5 mg of β-galactosidase for the whole vaccination cycle. Blood was taken on the 6-7th day after the last injection. Titer of antibodies was determined by the method of binary diffusion in agar.

To obtain gamma-globulin fraction the rabbit serum with titres 1 : 32 and 1 : 16 were two times precepitated by ammonium sulphate with 33 %-saturation, dialyzated against 0.0175 M of natrium-phosphate buffer, pH 6.3, and purified by passing through the column of DEAE cellulose (1.5×25 cm) with the same buffer being used. Fraction of IgG taken from the ion-exchange column was concentrated by PEG (35 000-40 000) and kept at 4 °C with added 0.1 % NaN_3.

Table 1

Used molecular constructions

Plasmid	Experiments they are used in
pGA293 *pGA293Z* *pGA293A* *pCH110* *pCMVβ* *pHβ21* *pHB320* *pLZ56*	Study of the expression of bacterial gene of β-galactosidase on the culture of cells and model animals
pBR322 *pBR322ins* *pPins* *pAins* *pGins*	Study of the expression of human insulin gene in the culture of cells and model animals
pAL1 *pAL1apo* *pAAA* *pUC18* *pUC18apo* *pUC18apo'* *pYF92*	Study of the expression of human gene *apoA1* on the culture of cells and model animals

Under immunofluorescent staining of cytological preparations (hepatocytes, Ltk⁻ and other cells) the IgG fraction was additionally purified during an hour by the hepatic powder [11].

The donkey antirabbit globulin labelled by fluoresceinisothiocyanate (FITC) was released from a nonbound fluorochromium on 1 % agarose. Working dilution of the rabbit serum to a bacterial β-galactosidase is 1 : 10 luminescent serum — 1 : 16 under staining of cytological preparation.

Determination of human *apoA1* in cell culture of mammals was performed by the standard immunoenzyme method [12] using rabbit antiserum to the human protein *A1* and conjugate on the base of horse-raddish peroxidase.

Producing of primary cultures of hepatocytes and keratinocytes. For production of hepatocytes we used the liver of 2-week rats. The liver was taken, washed in the Henks solution with antibiotics and ground. Then it was treated during 10 min by 0.02 % collagenase solution, pipetted and precipitated during 35 s under 1000 RPM. Cells were inoculated into the Petri dishes by 2 mill and placed into CO_2-incubator at 37 °C. In three hours the detritus was removed from the Petri dishes and the medium was changed. When the monolayer was formed the cells were once again inoculated and then again transfected.

Keratinocytes were grown from skin slices taken by biopsy using feeder layer. Cells 3T3 formed by gamma-rays were used as feeder cells. Skin slices were cut in the Eagle medium with 10 % of serum and then ground. Then they were treated with 0.25 % solution of trypsin at 37 °C. Each 30 min we let the skin crumps precepitate during 1 min with following removal of supernatant fluid and its replacement by fresh trypsin.

Then the cells were precipitated from the supernatant fluid and resuspended in the growing medium containing 20 % of serum from cattle embryo and hydrocortisone (0.4 μg/ml). This procedure promoted growing of the cells. After this the epidermal cells were mixed with the suspension of feeder cells and put into the dishes. Epidermal cells needed 2-3 days for set fixation. The medium was changed twice a week. Growth of fibroblasts in these conditions was supressed while the epidermal cells formed colonies of keratinocytes lining the feeder layer towards periphery.

For replating of keratinocytes the culture was treated by 0.02 % EDTA during 15 s and intensiveiy pippeted for a selective removal of fibroblasts. Then the keratinocytes colonies were desagreggated by 0.02 % EDTA and 0.05 % trypsin (1 : 1). New feeder layer was added for reinoculation in case of need.

Transfection. Transfection of cells by plasmids with the insulin gene was performed by the calcium-phosphorus method [13]. Concentration of the transformed DNA was 10 μg/ml for 1 mill of cells. In some experiments we used the herring milt DNA (commercial preparation). Calcium-phosphate precepitate was applied on the subconfluent state cells usually after 2-3 days after their inoculation into the glass flasks. Cells subjected to mock-injection and cells injected with initial plasmids used for construction of recombinant DNA acted as a control. The cells were cultivated in the Eagle medium with addition of 10 % bovine serum either embryonal or from adults. The content of the protein product was determined in the samples of the cultural medium by the immuno-enzyme method. With this aim we made probes during 16 days after the transfection and in the process of growth of the transfected cells. Before sampling the cells were washed twice by the Eagle medium and a portion of a fresh medium with an increased glucose content (1800-2000 mg %) but without serum was added. In a day after this procedure we collected medium from each flask separately and determined a concentration of the protein-product. Concentration of glucose in the samples of cultural medium was determined by the standard *o*-toluidine method. Statistical processing was performed by the Fischer, Vilkockson and $\chi 2$ criteria.

Transfection of Ltk$^-$ cells by plasmids containing *lacZ* gene under different promoters is carried out for cytological studies on 1-2 day cell culture inoculated on the sigmental glasses. Cells were fixed in 4, 24 and 72 h after transfection during 20 min by some mixtures of acetone cooled to 4 °C.

The recombinant plasmids *pAins* and *pGins* carrying human insulin gene were used in the experiments on animals. DNA was implanted into the experimental animals in a composition of liposomes prepared from a mixture of lipids: lecitin-cholesterin-dicetylphosphate (7 : 2 : 0.5). A day before the experiment we enclosed recombinant DNA into the liposomes, prepared suspension of the liposomes in the physiological solution, freezed and then unfreezed them directly before the injection. Suspension of the liposomes (75 μg of DNA in 0.2 ml per individual) was injected directly into the right side of the liver after the autopsy of the peritoneal cavity with a subsequent putting in stitches on the muscle layer and skin under ester narcosis. Empty liposomes and physiological solution acting as a control were injected the same way. Intraperitoneal injection is also used.

The content of glucose in blood was determined by the glucosooxidase micromethod on an empty stomach using the device Glucofot. Immunoreactive insulin and *C*-peptide were determined by radioimmune method using the device rio-INS-PGU designed at the Institute of Bioorganic Chemistry of the Academy of Sciences of Byelorus. The content of glucose in blood was determined before the injection of the preparations and then in dynamics in 6, 10 and 24 h after injection.

Experiments *in vivo* were performed on mice, rabbits, piglets and rats. For each variant of the experiment we prepared a group of 2 month mice of BALB/c line (4—5 specimens). Each experiment was repeated 4

times of the mean. The material was injected into a large lower lobe of the liver. Localization of the necessary part of the liver was found through a section of the skin integumentum at a level of the organ. The performed operations produced no noticeable effect on the animals.

Volume of the material injected into the mice liver was in all experiments 60 μl per specimen. In case of the injection of the plasmid enclosed into the liposome the injected material was 300 μg of lipids and 10 μg of plasmid DNA. Besides, injections of plasmid *pGA293* without liposomes and only nonloaded liposomes were made. Injections of physiological solution (10 mM tris-HCl, pH 7.5) were made to verify the adequate reactions of animals on administration of the exogenic material. Intact mice were control to the performed variant of the injection.

Intracutaneous injections of plasmid *pGA293* in composition of the liposome were made into the inguinal region of mice and 14-day piglets. A volume of the injected material and a correlation of plasmid DNA and lipids remained the same. 14-day piglets were injected with a mixture containing 3 mg of plasmid DNA and 20 mg of lipids, each by 2 ml.

Implantation of the genetic material with gene *apoA1* to experimental animals — adult (6-8 months) and old (4.5-5 years) rabbits as well as adult (6 months) and old (26-28 months) rats was carried out a direct injection into the liver. For injection we used 0.3 ml of liposome suspension for each rabbit per 1 kg of the live weight while for the rats — 0.1 ml of liposome suspension for 100 g of weight containing 13 mg of lipids and 400 μg of plasmid DNA in 1 ml of suspension. Expression in the implanted gene was estimated by means of the rocket immunoelectrophoresis of the blood plasma of experimental animals with a specific antiserum to human *apoA1* and subsequent quantitative estimation of the protein level by the control human serum containing 1.25 mg/ml of *apoA1* [14]. Total cholesterin was determined in the blood plasma by the Abell, Kiryakov and Tinterova [15, 16] while a per cent content of the fraction of lipoproteids — by the method of electrophoresis in the agarose gel by Kiryakov [17].

Analysis of lipoproteids. LP of different classes are isolated by the method of preparative ultracentrifugation in the density gradient of NaBr [18]. The content of protein [19], total cholesterin (ChS), ChS esters [20], phospholipids (PhL) [21] was determined in LP after preliminary extraction.

Protein composition of $HDLP_2$ and $HDLP_3$ fraction was studied by means of electrophoresis in the gradient of a polyacrylamide gel (PAAG, 3-27 %) in presence of DS-Na in the Na-phosphate buffer by the method described in [22]. PVP_2 and $HDLP_3$ proteins after delipidization by a chloroform-methanol mixture were dissolved in the buffer [23] containing 0.125 M of tris-HCl, pH 6.8, 5 % β-mercaptoethanol, 2 % DS-Na. Then they were heated during 2-3 min in the boiling bath and applied on the cells of a vertical PAAG block (16×18×2) by 30 μl (30 μl of a total protein). Electrophoresis was carried out at 25-30 °C during 15-18 h. Gel was fixed, stained with the 0.1 % Kumassi C250 and decolourized to reveal electrophoretic zones. The gels were densitometrized on the registering microphotometer IFO-451.

Microsomal fraction of the liver was isolated by the method of differential centrifugation at 105 000 *g* [24]. The content of cytochrome P-405 was determined by the method of two-ray differential spectrophotometry on the spectrophotometer «Perkin — Elmer» [25]. Monooxygenase activity of microsomes was revealed *in vitro* by adding hydroxylation substrates — amidopyrine and aniline [24].

Preparation of hepatocytes. Mices were mortified temporary in 1, 2, 3, 5 and 8 days under injection. For cytological preparations we used the part of the liver to where the material was injected. The finely-divided liver was washed of blood elements, kept at least 30 min in the cold Na-acetate buffer (0.88 % NaCl, 0.7 % Na-citrate, 0.1 % glucose, 5 mg/ml of heparine, pH 7.4) and used for hepatocyte smears. Quality

and density of the smears were checked under the light microscope. The smears were fixed during 20 min by the acetone cooled down to 4 °C. Morphological preservation and functional activity of hepatocytes were checked on particular preparations stained by 0.2 % trypsin blue. Yield of sound nondamaged cells of hepatocytes varied within 90-95 %.

Skin preparations. For obtaining epithelium of the piglet's skin the small grafts of skin (5×5) were ground and washed in the Henks medium. Then the material was placed into the Versen mixture (trypsin 1 : 1) and kept during 18-20 h at the temperature 4 °C. When the trypsin-Versen mixture was changed the containers with the ground skin were macerated on a magnetic mixer during 30 min. Epithelial cells with fibroblasts were isolated from the admixture of tissue remains by centrifugation at 1000 RPM during 15 min. Cells were applied on the subject glass and after fastening they were fixed in three mixtures of cooled acetone during 20 min.

Study of cytological preparations. The expressions product of *lacZ* gene in mice hepatocytes was tested by indirect immunofluorescent method (IF) [26]. The preparations were studied on a luminescent microscope ML-2 with a photometric attachment under the wave length 520 nm with a probe 0.5 μm. The objective lens X70 for water immersion were used for examination of the preparations. A stained product of *lacZ* gene expression fluoresced green in UV light. Glow intensity of each separate hepatocyte was determined as a mean value from the probe measurement of three points in different sites of the cell cytoplasma. Measurements for each experiment were made for at least 30-50 hepatocytes with 4-5-fold repetition (on parallel preparations). The presence of bacterial β-galactosidase in the transfected Ltk⁻ cells and hepatocytes was also detected by other cytochemical method-staining of preparations with ONFG [27].

Induction of streptosotocyne diabetes in Wistar rats. Preliminary we developed a model of insulin-dependent diabetes in Wistar rats using preparation streptosotocyne synthesized for this purpose at the Institute of Molecular Biology and Genetics of the Academy of Sciences of Ukraine. Optimal conditions for inducing diabetes were as follows: doses of preparations from 55 to 70 mg per 1 kg of the live weight depending on the season. Streptosotocyne before administration (not earlier than 30 min) was diluted in the acidic citrate buffer at pH 5.0. Males weighing 150-180 g were used for the experiments. It was established that single administration of streptosotocyne induced diabetes with a different degree of manifestation by glucose induces in blood and urine. Insulin-dependent diabetes became stable in 3 weeks after administration of streptosotocyne in more than 70 % of animals. IDDM manifested itself with a different degree — from light glycemia to the death of animals. It testifies to the individual sensitivity to the preparation. Some animals did not fall ill.

Results and its discussion. Search of approaches to fundamental solutions on model objects in vitro. The first stage is devoted to search of specific methods for solution of problems connected with the first peculiarity of mass pathologies. It embraces search and analysis of such regulatory elements that would permit an expression irrespective of the state of a general cell regulation. It is necessary to check up different molecular constructions in different types of cells. Studies are carried out on model objects using genes that are key ones for mass pathologies. Gene of β-galactosidase in *E. coli* is chosen as a marker (reporter) one. It is well differentiated by antibodies of other cell proteins while cytoimmunochemistry and in particular cases enzymatic catalysis as the methods of its identification are well developed.

An expression of genes in different types of cells (*in vitro* — HeLa, Ltk⁻, primary cultures of hepatocytes and keratinocytes, *in vivo* — hepatocytes, skin epithelium) is analyzed using well-studied promoters both tissue-specific and nonspecific. The first data have shown that even ho-

mogeneous population under standard well-controlled conditions produces high heterogeneity by the expected response. Ability of *lacZ* gene in composition of different vector molecules to express bacterial β-galactosidase in the culture of mice fibroblasts and HeLa cells is tested by cytochemical methods using indirect immunoenzymatic (IE) analysis. Con-

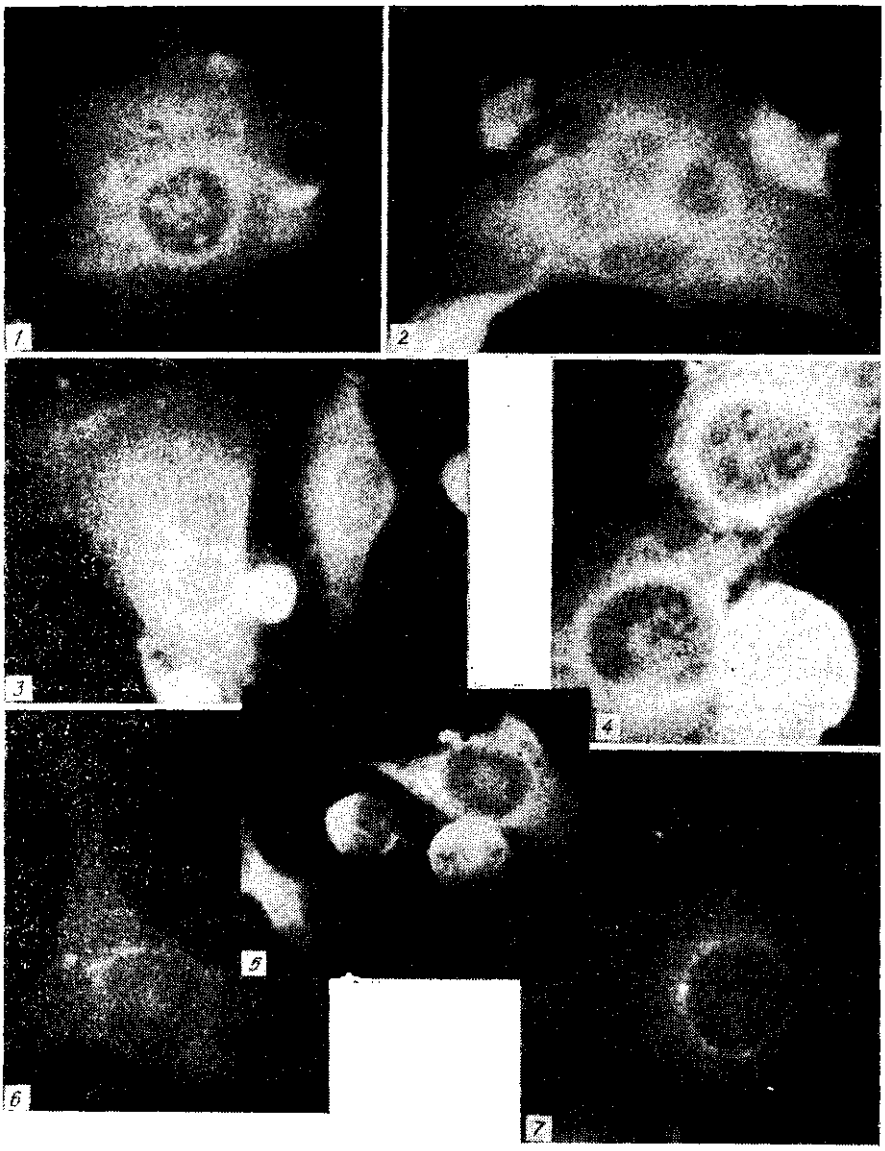


Fig. 3. Immunofluorescent of *Ltk*$^-$ cells after transfection with plasmids: *1* — *pGA293*; *2*, *4* — *pGA293A*; *6* — *pGA293Z*$^{\Delta}$; *3*, *5* — *pCH110*. 4 h after transfection *(1—3)* and 24 h *(4—6)*. Control *(7)* nontransformed cells

structions of the main recombinant molecules used in the work are presented in the section «Material and methods».

As early as in four hours after transfection of Ltk$^-$ cells by plasmids *pCH110*, *pGA293* and *pGA293A* one observes a bright fluorescence of cytoplasma in the green spectrum that testifies to the appearance of the bacterial β-galactosidase (fig. 3, *1*, *2*). Curves for luminous emittance distribution of cells after transfection with these plasmids is presented in fig. 4. In control variants of tests when the culture of mice fibroblasts is treated with *pGA293Z*$^{\Delta}$ plasmids and in intact cells one observes no pronounced fluorescence of cytoplasma (fig. 3, *6*, *7*). Fluorescence inten-

sity of fibroblast cytoplasma essentially increases in 24 h after transfection. It testifies to the accumulation of β-galactosidase, a tested enzyme, in it (fig. 3, *4*, *5*). When using *pGA293A* and *pCH110* constructions a level of the luminous emittance in cell cytoplasma is always higher both in 4 h and in 24 h in comparison with plasmid *pGA293* [28, 29].

Qualitatively similar pattern is observed under comparative study of the bacterial β-galactosidase content after transfection of fibroblasts by plasmids with *lacZ* gene under promoters of virus HbsAg of hepatite B and earlier promoter *SV40* [29, 30]. Cytoplasma of control cells (cells are treated with «empty» liposomes) in 72 h after transfection has

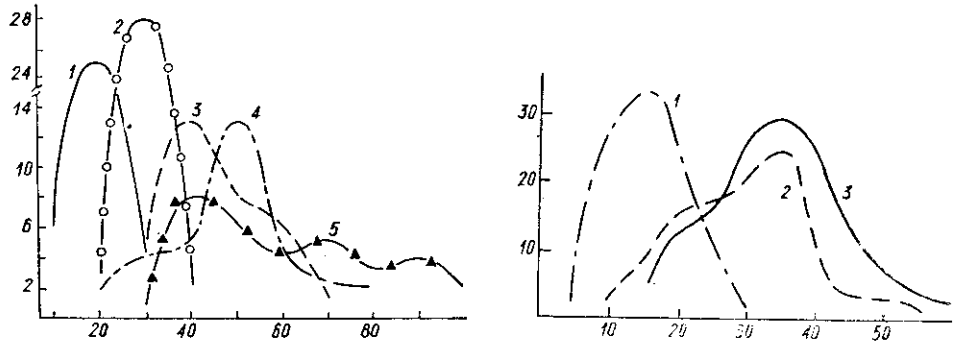


Fig. 4. Transfection of *Ltk*⁻ cells: *1* — control of staining; *2* — control (nontransformed cells); 4 h posttransfection: *3* — *pGA293*; *4* — *pGA293A*; *5* — *pCH110* (there and on the figs 3—9 ordinate axis — the number of cells with a definite intensity of fluorescence; absciss axis — fluorescence intensity of cells in relative values). Peaks marked on the plot correspond to the largest quantity of cells with the given luminous emitence

Fig. 5. Distribution of *Ltk*⁻ cells by fluorescence intensity. In 72 h posttransfection: *1* — treatment with «empty» liposomes; *2*, *3* — DNA plasmid *pCH110* and *pHβ21* respectively in composition of liposomes

a weak glow, nuclei do not fluoresce. Distribution of fibroblasts by fluorescence intensity corresponds to the normal distribution (fig. 5). Cells fluoresced by plasmids *pCH110* and *pHβ21* display another fluorescence pattern. In 72 h after transfection one observes the appearance of fibroblasts with vividly fluorescing cytoplasma in their population. When the curve of the test variant distribution is broked down into components two main curve peaks corresponding to different fluorescence intensity are distinguished. The first peak appears to be nontransfected cells or the cells where *lacZ* gene does not manifest itself by some reason. The second peak corresponds to intensively fluorescing cells. Bright specific fluorescence of their cytoplasma testifies to an expression of gene by bacterial β-galactosidase. Pattern of the curves describing fluorescence intensity of mice fibroblasts transfected by plasmids *pCH110* and *pHβ21* are noted to be similar. It evidences for about similar level of expression of *E. coli* gene under promoters HbsAg of hepatitis virus and en earlier promoter of virus SV40 in mice fibroblasts.

Statistical processing is presented as data on a mean fluorescence intensity of cells depending on the type of the introduced DNA plasmids (sampling for each variant is $n=60$). Confidence limit of mean values and assurance of the boundary between control and test curves are determined for the probability $n=0.999$:

Intact cells	17.93±0.714
After transfection DNA of plasmids:	
pCH110	29.20±1.38
pHβ21	31.55±1.52

Therefore, differences in fluorescence intensity of cells transfected by DNA of plasmids *pCH110* and *pHβ21* are inconsiderable. However differences in glow, i. e. in the expression level of the studied gene are great in different cells of one population.

Expression of model *lacZ* gene of *E. coli* in composition of vector molecules (plasmids *pGA293. pCMV*β) controlled by the earlier promoter SV40 as well as in plasmid *pH*β*21* under promoter of HbsA$_B$ hepatitis virus B is carried out in a sequence of experiments with a primary culture of human and rat hepatocytes [31]. Transfection is performed by Ca-phosphate method. Expression product of bacterial β-galactosidase is tested in 4, 24 h, in 2 and 4 days [32].

Transfection of the primary culture of rat's hepatocytes by plasmids *pGA293A, pCMV*β and *pH*β*21* is carried out in one experiment. Fixed culture of cells are stained simultaneously by indirect IF method. So,

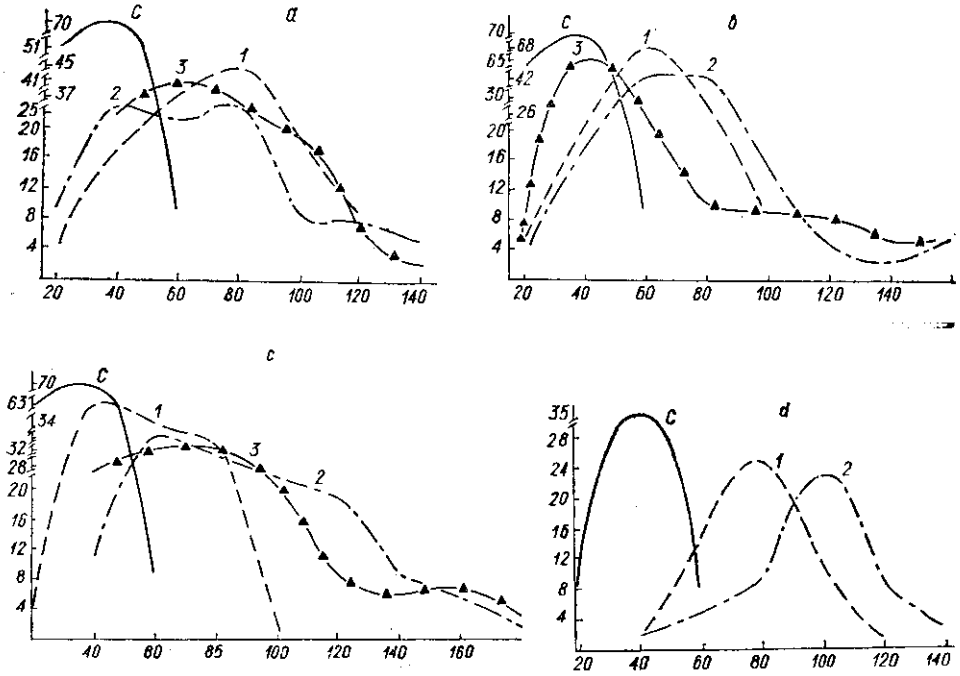


Fig. 6. Transfection of the primary culture of rat hepatocytes with plasmids: *a* — *pGA293A*; *b* — *pCMV*β; *c* — *pH*β*21* (*C* — control; *1* — 1 day; *2* — 2 days; *3* — 4 days after transfection); *d* — 2 days after transfection of human keratinocytes by plasmids: *1* — *pGA293*; *2* — *pCMV*β; *C* — control

the obtained results are correctly comparable as to an expression level of each plasmid in a definite periods of time. Independent experiments conducted in different time show that quantitative values (brightness of luminescence, percent of cells with identical luminous emittance) are usually somewhat different with the same plasmids and the cells of the same type. A level of expression and, thus, the quantity (in relative values) of the β-galactosidase enzyme developed by the cells is very convenient to trace for each particular plasmid in a day, two, four and so on under the used method for construction of plots. On the other hand it is possible to compare these three plasmids by a level of β-galactosidase expression in different periods. Proceeding from the presented plots (fig. 6, *a*, *b*) it is seen that β-galactosidase in case of hepatocytes is tested in a day in large quantities of cells when using plasmids *pGA293A* and *pH*β*21*. In two days the most of all brightly fluorescing cells are observed in the variant with *pH*β*21*, the least of all — after transfection with plasmid *pCMV*β. In a case of plasmid *pGA293A* the quantity of the transformed cells is the lowerest. A similar result is obtained in tests on human keratinocytes (fig. 6, *d*). The quantity of the transformed cells when using plasmid *pGA293A* in two days is smaller than with plasmid *pCMV*β. On the 4th day from transfection the quantity of cells of a primary culture of rat's hepatocytes containing bacterial β-galactosidase in the variant

with *pGA293A* is almost the same as that after one day from transfection. In the variant with *pCMV*β it falls to the level of control, in case of *pH*β*21* it is about the level observed on the second day. However particular cells with brightly fluorescing cytoplasma occur in culture thus indicating an increase of β-galactosidase content.

We perform also independent experiments on determination of *lacZ* gene expression by biochemical methods to support the results. Activity of bacterial β-galactosidase is determined by biochemical methods by the splitting efficiency of O-nitrophenyl-β-D-galactoside substrate. Activity of β-galactosidase in Ltk⁻ cells is determined on the third day after transfection. The results are presented in table 2.

As is known eukaryotic cells have their own β-galactosidase activity. However, introduction of *E. coli lacZ* gene leads to a considerable increase of a level of bacterial β-galactosidase in them. Expression efficiency of *lacZ* gene in composition of *pH*β*21* plasmid is the same or somewhat higher in Ltk⁻ cells transfected by *pCH110*. It supports the data obtained by the cytochemical method (see fig. 5).

Determining β-galactosidase activity by the splitting efficiency of O-nitrophenyl-β-*D*-galactoside we estimate a total activity of the given enzyme that is composed of the cell's own β-galactosidase activity and bacterial β-galactosidase introduced into the cell.

It should be also taken into account that β-galactosidase gene due to the peculiarities of the initial plasmid structures is not complete. It lacks a part of the sequence from 3′ end. That is why its enzymatic and antigenic activity does not quantitatively coincide. However qualitatively the data obtained by IF method and by a direct analysis of the enzymatic activity have the same tendency.

Thus, tests on preparations have shown that a theoretical prediction of the expression pattern of gene in composition of different vectors when implanted into different types of cells proves unreliable. We may only say in the first approximation that (for the given systems) hepatocytes and keratinocytes are better than fibroblasts. Besides it is evident that the cell population will be heterogeneous by the final effect — the expression of the implanted gene. Finally, there is no assurance that the regularities obtained on the cell cultures (i. e. outside the organism) will prove legitimate even for the same cells in the organism. So, the second level of approximation (approaching human) may be only animals. It is necessary to estimate the expression pattern of the implanted gene in cells within the organism.

Search of approaches to fundamental solutions on model objects in vivo. Considering the obtained data hepatocytes are chosen as cells-targets, while a direct injection of the recombinant molecules in the composition of liposomes as a system of administration [33, 34].

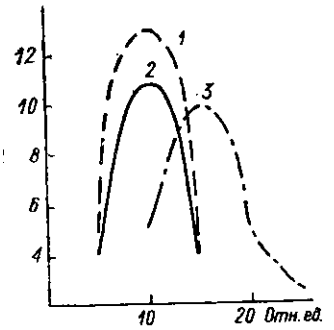
Table 2

Comparative study of β-galactosidase content in Ltk⁻-cells, transfected plasmid DNA containing lacZ gene under different promoters

*pH*β*21*, ME/ml	*pHB320*, ME/ml	*pCH110*, ME/ml	Cells Ltk⁻ without plasmid, ME/ml
2.4	1.4	2.4	0.63
3.5	2.2	3.5	0.72
1.6	0.82	1.7	0.52
2.4	1.18	2.5	0.318
6.6	—	5.2	1.48
6.4	2.4	4.8	1.6
		$M \pm m$	
3.5±0.52	1.6±1.3	3.3±0.5	0.87±0.22

Liposomes as suppliers of plasmid DNA directly into the animal's organ (liver, skin) are chosen because they facilitate DNA conveyange into the cells and protect it from degradation (composition of liposomes and the method of their preparation see in the section «Materials and methods»). Besides a direct injection into the tissue allows the material to be delivered directly to cell membranes. Intravenous administration will inevitably calls forth the problem connected with a vessel barrier. Vessels of all levels including capillaries are rather an effective barrier that prevents penetration of many components through them.

The work with hepatocytes *in vivo* have engaged our attention to the background glow of their cytoplasma (autoluminescence) in the green



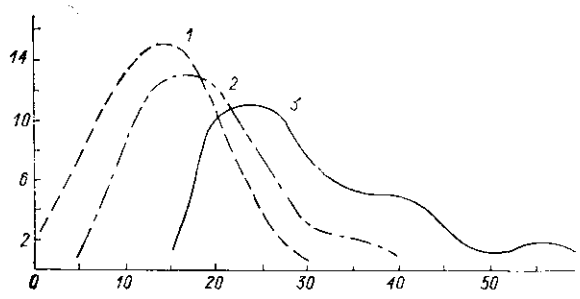


Fig. 7. Autoluminescence of mice hepatocytes: *1* — intact animal; *2, 3* — in 24 h after injection of «empty» liposomes and plasmid *pGA293* with liposomes into the liver

Fig. 8. Distribution of mice hepatocytes by the fluorescence intensity: *1* — intact animals; *2* — 24 h after injection of plasmids *pGA293Z*$^{\Delta}$ and *3* — *pGA293* in liposomes into the liver

spectrum where bacterial β-galactosidase is tested as a product of *lacZ* gene expression. It may distore the final results without considering the given phenomenon. The results from measurements of hepatocyte's own luminescence (i. e. without any additional treatment) after administration of pure liposomes and plasmid *pGA293* into the liposome are presented in fig. 7.

Fluorescence of mouse hepatocytes from control groups (administration of nonloaded liposomes, physiological solution) in 4-24 h after injection is at a level of luminous emittance in intact animals. A lower level of cytoplasma fluorescence is also observed after injection of plasmid *pGA293Z*$^{\Delta}$ (without *lacZ* gene) with a liposome. However in the latter case one observes an inconsiderable shift of the distribution curve maximum towards larger intensity of fluorescence with a marking tendency to deviate from the normal distribution: part of cells outside the Gaussian curve possesses brighter fluorescence (fig. 8, *1*, *2*). Hepatocytes of animals whose liver is injected with plasmid *pGA293* containing *lacZ* gene display the largest shift of the maximum (among control variants) on the distribution curve towards larger fluorescence intensity [33, 34].

In 24 h after administration of plasmid *pGA293* into the liposome one observes the appearance of cells with brightly fluorescing cytoplasma in population of hepatocytes (fig. 8, *2*). In one of the tests we break up the curve into the components. It allows revealing two main peaks corresponding to different fluorescence intensity (fig. 9, *a*). A group of cells with the brightest fluorescence makes up about 5 % of the total number.

The first peak corresponding in its form to luminous emittance distribution of hepatocytes in the control (intact animals and in the variant with injection of DNA *pGA293Z*$^{\Delta}$ to 75 % of cells) presents, apparently, either cells not at all transfected by plasmid or transfectants where plasmid is not expressed by some reason (fig. 9, *b*). The second peak (about 22 % of hepatocytes) accounts for the cells with bright specific fluorescence. The appearance of 5 % hepatocytes with the brightest luminous

emittance may be attributed to the peculiarities of the metabolic state of some liver cells that provides the most efficient expression of *lacZ* gene (fig. 9, *c*). A control treatment of preparation by nonspecific rabbit's serum testifies to the absence of a specific fluorescence. Thus, a nonsimilar fluorescence of hepatocytes under administration of plasmid *pGA293* into liposomes may be considered as a consequence of heterogeneity (cells by a level of synthesis in them) of bacterial β-galactosidase.

The considered results may be presented also as data on a mean fluorescence intensity of hepatocytes (arbitrary units) in 24 h depending on the type of the injected material (sampling for each variant is 220

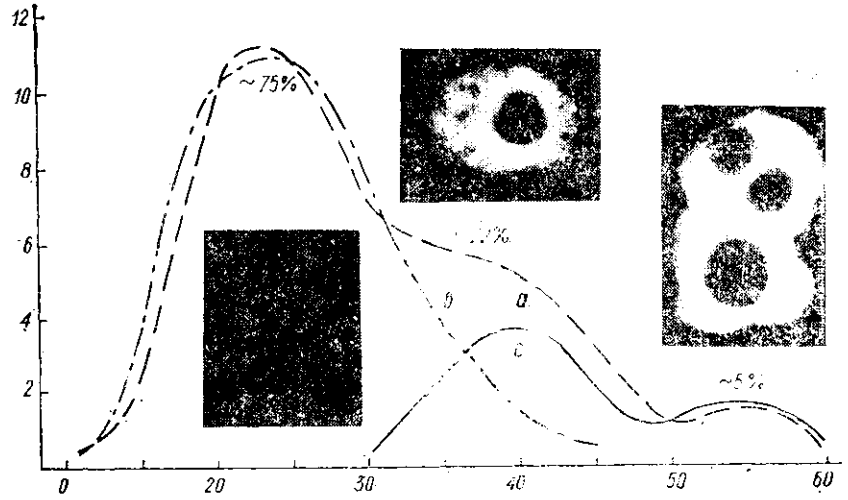


Fig. 9. Example of the analysis of distribution curves by fluoresglow intensity (24 h after administration of plasmid *pGA293* in liposomes). Explanation in the text

cells). Confidence limits of mean values and reliability of the difference of two peaks on the experimental curve are determined for the probability ±0.99. Population of cells with the brightest fluorescence is not presented here because of its small number.

Intact animals	14.5±4.2
After injection of plasmid *pGA293Z*Δ	18.1±3.8
The same *pGA293*:	
I peak	25.7±3.8
II peak	40.5±3.3

We have also experimentally studied preservation time of *lacZ* gene expression products after introduction of a construction *pGA293A* into liposome. These experiments show that the enzyme is continued to be tested in hepatocytes during 8 days (fig. 10). Since the used plasmid does not integrate in gene the sequence *olu* is likely to perform here either promoting or enhancing functions that leads to a large accumulation of *lacZ* gene product. However the quantity of brightly fluorescing hepatocytes is noticeably lower than in a day after the injection. Fluorescence of the bulk of cells does not differ from that when using plasmid *pGA293Z*Δ in composition of liposomes (fig. 10), i. e. with no β-galactosidase in them.

Besides heterogeneity of cells one observes also individual differences on the organism level by an expression criterion of the introduced gene.

A distinct individual reaction of mice on the injected materials is observed during the experiment with animals. As a rule, about 50 % of animals either do not react on the administration of the plasmid material or there is a very weak expression of *lacZ* gene in their hepatocytes. β-Galactosidase in the liver of other animals is tested in quantities sufficient to be tested by indirect IF method. The appearance of the indivi-

dual response in 8 days after injection is revealed more clearly than in experiments where the product is tested in a day or two. The quantity of animals with the expression product of *lacZ* gene detected in 8 days becomes ever smaller then the animals whose expression is observed in 2 days.

The presence of β-galactosidase in hepatocytes is also confirmed by other cytochemical method — staining of the cells by OHFG. The reaction product as small granules is distributed by the cell periphery in hepatocytes of intact animals. Smaller quantities are observed in the central part of cytoplasma. Heterogeneity of cells by β-galactosidase activity is

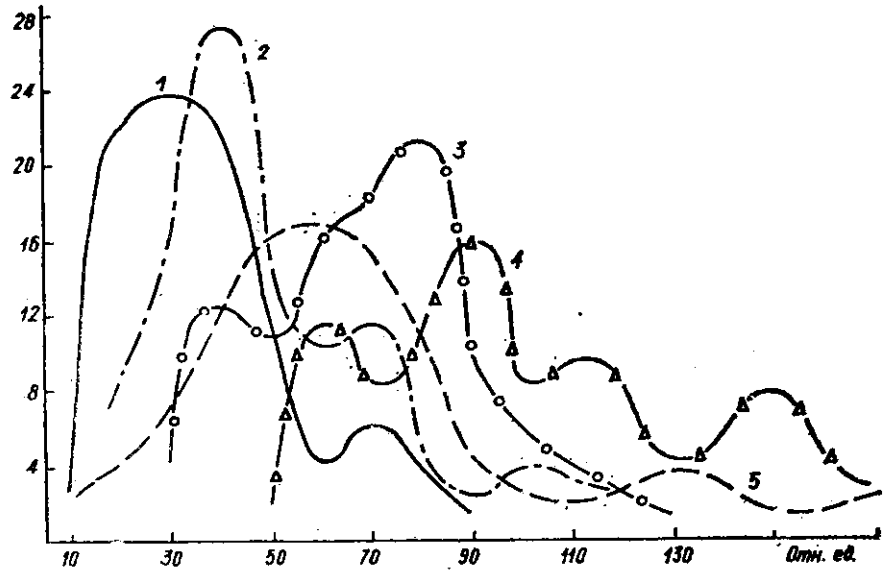


Fig. 10. Distribution of mice hepatocytes by a fluorescence level in 24 h after injection in liposomes into the liver: *1* — *pGA293ZΔ*; *2* — *pGA293*; *3* — *pCH110*; *4* — *pGA293A*. In 8 days after administration: *5* — *pGA293A*

pronounced more distinctly in test variants than in control one. Dense accumulations of the expression product of β-galactosidase activity appear in the majority of cells closer to the cell periphery. Particular granules of the product locate near nuclei or freely in cytoplasma. Distribution of the product with enzymatic activity in some cells does not differ from that in hepatocytes of control animals.

Somewhat different data are obtained when using vector on the base of hepatitis B virus DNA [30].

Studies of the *lacZ* gene expression in plasmid *pHβ21* and *pCH110* administrated into the liver of monthly mice have shown a tissue specificity of the vector obtained on the base of hepatitis B virus DNA. As in the above experiments the transfected hepatocytes have differently fluorescing cytoplasma and dark nuclei. The quantity of the transformed cells under the use of *pHβ21* plasmid in composition of liposome is in 24 h after the injection much higher than in experiments with *pCH110*.

One is inclined to think that the presence of hepatitis B virus enhancers in plasmid *pHβ21* promotes an increase of *lacZ* gene expression in animals' hepatocytes. Comparison of this gene expression in plasmid *pHβ21* in culture of mice fibroblasts and in liver hepatocytes testifies to tissue-specificity of the hepatitis B virus enhancers. In animals this specificity manifests itself stronger than in the cell preparations.

Liver is convenient but not the only organ suitable for administration of recombinant molecules. A possibility to administer recombinant molecules intraperitoneously is studied as an example. Piglets are taken as an object of investigation.

Administration of foreign genetic material intracutaneously to 14-day piglets allows testing bacterial β-galactosidase in the epithelial cells in a day after the injection. Nonloaded liposomes serve as a control to injection of plasmid *pGA293A* in composition of liposomes (fig. 11). Less than 10 % of epithelium cells correspond to the control variant as to the glow intensity while the bright fluorescence of cytoplasma in the rest of the cells testifies to the presence of the bacterial β-galactosidase. Possibly so high efficiency of the transfection is connected with the age of animals.

Therefore, expression of the implanted gene material is shown on the organism level on cells of different tissue (and in different organisms)

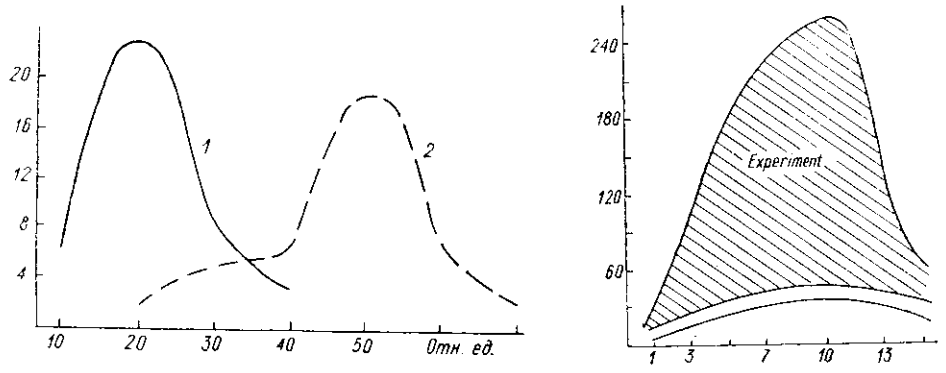


Fig. 11. Immunofluorescence of skin epithelial cells in 14-day piglets in 24 h after injection: *1* — nonloade «empty» liposomes; *2* — plasmid *pGA293A* in liposomes

Fig. 12. Content of insulin-immunoreactive proteins in the samples of culture medium in different terms after transfection of fibroblast cells in mammals (ordinate axis — protein, mg/ml; absciss axis — days)

These works allow due to a possibility to estimate expression of the marker gene in cells establishing a heterogeneity of cells by the studied sign as well as a character of this heterogeneity. Besides it is found out that heterogeneity is intrinsic in animals. It should be taken into account in the work. In future it is necessary to design a test-system that allows estimating individual perceptibility of the organism to recombinant DNA. Such estimation must be followed by optimization of the administration regime.

Knowing general regularities of the implanted gene manifestation and having the developed system of administration at hand we are coming now to the study of a fundamental possibility of gene therapy of mass pathologies.

As mass pathologies to study their parrying by the gene therapy we chose insulin-dependent diabetes melitus (IDDM) and atherosclerosis.

Substantiation of fundamental possibility of gene therapy of insulin-dependent diabetes melitus. IDDM is a boundary between classical hereditary diseases and classical mass pathologies. On the one hand such duality is explained by the fact that hereditary component of IDDM appearance is already known though not in all cases but rather often. Moreover, it is formally monogenic in the sense that the disease is connected with an absence (or lack) of the product from only one gene — the one which codes insulin. It is formally monogenic because insulin is synthesized in the quantity necessary for the organism only in pancreas β-cells. Death of these cells leads usually to a lack of a total quantity of hormone. However the differentiation of β-cells, i. e. formation of cells synthesizing insulin is under a very complex genetic control that is not yet wholly identified and with many genes participating in it. On the other hand IDDM is really a mass pathology.

In some rare cases this disease is classical hereditary, i. e. «from birth» and is connected with a genetic defect in the insulin gene. But in most cases it is induced by the insulin deficiency though the gene that

codes insulin in all cells of the organism is not damaged. But rigorous tissue specificity of the expression blocks its activity even if the organism is agonizing because of its lack.

According to some data diabetes takes the third place among the mortality causes in the developed countries. Recently one observes growth of diabeties rate. IDDM makes up the bulk of such type of diseases. Under juvenile diabetes one observes, as a rule, an early degradation of β-cells producing insulin in the organism [35]. IDDM conditioned by the point mutations in a structural part of the insulin gene [36] proceeds phenotypically the same.

In these cases patients are treated by administration of considerable doses of insulin obtained either by isolation of corresponding protein from the pancrease of mammals or by biotechnological methods.

At the same time radical treatment, i. e. elimination of the causes of this disease may be provided only by gene therapy. In this case it is possible to administer insulin-producing cells in capsules or in some other way into the patient's organism or to implant a normal insulin gene in such molecular construction that provides its expression in patient's cells including nonspecialized cells. For development of new approaches to treatment it is important to study expression of the exogenic insulin gene in the isolated cells of mammals *in vitro* and in cells from tissues *in vivo*.

Insulin-coding gene is located in the eleventh chromosome in human [37]. It is established at present that regulation of the insulin gene expression is provided by 5′-flanking region that included unique sequences and tandem repetitions [38, 39]. It is this region that determines high tissue-specificity of the expression (only β-cells of the pancreas). Therefore for expression of the insulin gene in the cells where it does not work one usually uses a standard technique of connection of a structural part of this gene to some other regulatory sequence. This technique was used to demonstrate expression of the human insulin gene in the cells of CV-1 and COS lines [40, 41] when it was implanted into the cells with SV-40 and Ad2 regulatory elements.

In our work we solve the following problems: study of the expression of the genome gene of human insulin in composition of different molecular structures, study of the peculiarities of its expression in the cultivated cells of different origin, development of the method for selection of cells transformed by the insulin gene and study of possibility to express the insulin gene in tissue cells under its direct implantation in liposomes into the organism of the experimental animals.

A possibility of the expression of genome gene of human insulin from its own promoter in absence of the regulatory site providing tissue-specificity of the expression is studied in a detail in cultivated fibroblasts of human and mouse. Results of twenty one independent experiments are described in previous works [42-44]. It should be noted that rather large variations in composition of the protein-product in the cultural medium are observed in the experiments, though the experimental conditions are thoroughly standardized. Such variations are attributed particularly to fluctuations under formation of Ca-precipitate that is a defining function of the transfection level. It must be considered when using this method. However analysis of all the performed experiments allows tracing a dynamics of its secretion after administration of recombinant *pBR322ins* plasmid into the cultivated cells. Since there is not noticeable difference in a dynamics of the product in case of human fibroblast LEH and mouse C3H10T1/2 [42] the data are presented in one figure (fig. 12). Concentration of the tested protein (ng/ml) of the cultural medium is shown in the ordinate axis, time after transfection of cells — in the absciss axis.

In a day after administration of the recombinant DNA into the cell system a level of the studied protein does not reliably differ from the control. Beginning from the 3d and up to the 10th day one observes a gradual increase of the protein-product concentration in the cultural me-

dium ($p<0.001$). On the 13th day a level of the protein secreted by the cells sharply decreases. On the 16th day and later a concentration of the tested protein achieves 30-45 ng/ml. It must be noted that monoclonal antibodies selected by us for testing recognize the determinant common for insulin and proinsulin and possibly for insulin-like growth factor evolutionally approaching them. The fibroblasts are known to produce insulin-like growth factors in the process of normal reproduction therefore a relatively high background level in binding of antibodies takes place in control samples.

Expression of the genome insulin gene in the recombinant construction *pBR322ins* in fibroblasts of four cell lines: LEH, SL, C3H10T1/2 and Ltk$^-$ are compared. It is shown that in all cases a maximal concentration of the studied protein-product in the cultural medium amounts to 200-250 mg/ml. Transfection of SL fibroblasts by DNA of recombinant *pAins* containing *alu*-repetitions also results in a secretion of a corresponding protein-product into the cultural medium.

Concentration of the protein-product under administration of plasmid *pBR322ins* into cells of other types (embryonal mouse hepatocytes) amounts to 120 ng/ml, i. e. twice as lower as in the above experiments.

Cells of the human insulinome taken as a positive control produced into the medium 125 ng/ml of the protein-product under the same conditions. The product of the studied protein is investigated under considerably increased level of the glucose concentration (1800 mg %) as compared with the normal (100 mg %) that may increase a transcription and translation regulation. In case of insulinome the produced protein is almost completely presented by insulin, that is apparently approaching the maximum which may be obtained in the culture of nonspecialized cells (insulinoma as all tumours consists mostly of dedifferentiated cells).

To increase a level of synthesis we carry out a repeated transfection of three experimental preparations of LEH under the same conditions as in the first transfection. Then the content of insulin-like protein factors in samples of the preparations are compared after first and second transfection. In all three cases after the second transfection one observes a reliable increase of the protein-product as compared with the one obtained after single treatment. If after the first transfection a maximal concentration of the protein-product in the cultural medium is 140, 150 and 250 ng/ml, then after the second — 800, 250 and 400 mg/ml, respectively. There are essential individual differences in the protein-product between three preparations, though one and the same DNA preparation is used for transfection, the latter being carried out simultaneously under the same conditions. The glucose content in one and the same samples is studied for estimation of the functional state of the expression product of the insulin gene. Glucose concentration in the samples of the cultural medium of the same preparations (when the glucose consumption is insulin-dependent) is decreased by 370, 110 and 360 mg %, respectively in relation to the control level (table 3).

Table 3

Maintenance of insulin and glucose in samples of cultural medium

Samples	Total		Difference between treated and untreated variants	
	Insulin, ng/ml	Glucose, mg %	Insulin, ng/ml	Glucose, mg %
Untreated samples	30	1860	—	—
Treated samples:				
1	140	1490	110	—370
2	150	1750	120	—110
3	250	1500	220	—360

Therefore, the second transfection of the recombinant DNA *pBR322ins* cells in human leads to an increase of the concentration of the secreted protein-product. The secreted protein-product has a specific functional activity that results in a decrease of the glucose concentration in the cultural medium. An increase of the protein-product yield under repeated transfection is, on the one hand, connected with an increase of the insulin gene dose, on the other — with a selection of most competent cells from the general heterogeneous population by this index. As it is shown the used method of the immune-enzymic analysis does not allow determining which protein exactly is secreted-insulin or proinsulin, i. e. whether the protein processing proceeds in the studied cells of the culture. It is known from literature that only proinsulin is tested in cultivated cells of the monkey's kidney CV-1 containing the implanted insulin gene of human. There is no processing of proinsulin into insulin [40]. Proinsulin is known to possess a weak insulin-like action. We suppose that proinsulin is obtained in our experiments, since the glucose content with its daily decrease does not achieve a normal level (100 mg %). It may be also assumed that a part of immune-reactive protein is evolutionally similar insulin-like growth factors produced by fibroblasts. The obtained results support the following conclusions. Gene of human insulin without its regulatory site responsible for tissue-specificity may function in the cultivated fibroblasts of different origin. When studying fibroblasts in human and mouse one observes a similar time dependence of the protein-product secretion of the insulin gene after implantation of the transforming DNA. A level of the secreted protein is reliably increased as against control from 3d to 10 days and then it is sharply decreased. A maximal yield of the protein-product under one-time transfection is 200-250 mg/ml. Repeated introduction of gene increases the synthesis almost twice. A secreted protein is likely to be proinsulin that possesses a specific functional activity resulting in a decrease of the glucose concentration in the cultural medium.

A presence of a similar time dependence of the insulin gene protein-product secretion for fibroblasts of different origin testifies that such processes are not apparently species-specific. The next task of our work is to make an attempt to select cells transformed by the insulin gene devoid of a regulator of the expression tissue-specificity.

Klein's team has developed and evaluated the method of implantation and selection of the required hereditary information using cells from human and animal bone marrow for works on gene therapy [45]. This method is rather effective but as the same time it has evident drawbacks connected with a necessity to administer a toxic selecting agent and with a presence of a gene-assistent that inevitably contaminates the genome, thus inducing side biological effects.

We use another method for selection of the transformed cells. It is known that insulin-producing β cells are capable to fission. Insulin in combination with other growth factors and tumours promoters of TPA stimulates human and mouse fibroblasts for fission [46]. Thus, the presence of cells producers of insulin and/or proinsulin in the cell population after transfection can possibly create a selective advantage for growth and leads to enrichment of population with cells-transformants under passage. An increase of survivability and improvement of growth properties may be particularly pronounced in the medium depleted with serum or without serum.

To verify this supposition we carry out transfection of LEH cells by means of DNA *pBR322ins*, then inoculate them and cultivate during 10 days (time of expression) in the growth medium with 10 % of embryonal serum. After this we replace the growth medium by the maintenance medium with 3 % of the adult animal's serum and hold cells in these conditions longer than 1 month (5 weeks). It should be noted as against embryonal serum of cattle (60 ng/ml of insulin-like proteins) that serum of adult animals is depleted with growth factors (less than 1 ng/ml).

From 30 to 60 ng/ml of insulin-like proteins are observed in samples of the cultural medium during the studied period.

Then the cells are replated once a week to determine the protein-product directly before each following passage. The results obtained under determination of the content of the protein-product of the insulin gene is subcultures of LEH cells are presented in fig. 13. The content of protein after first and second passage exceeds 100 ng/ml. Further one observes variations of the protein-product content in the samples of five studied subcultures of human cells, but generally a tendency to an increase of protein secretion to the level of 200-250 ng/ml is traced (fig. 13).

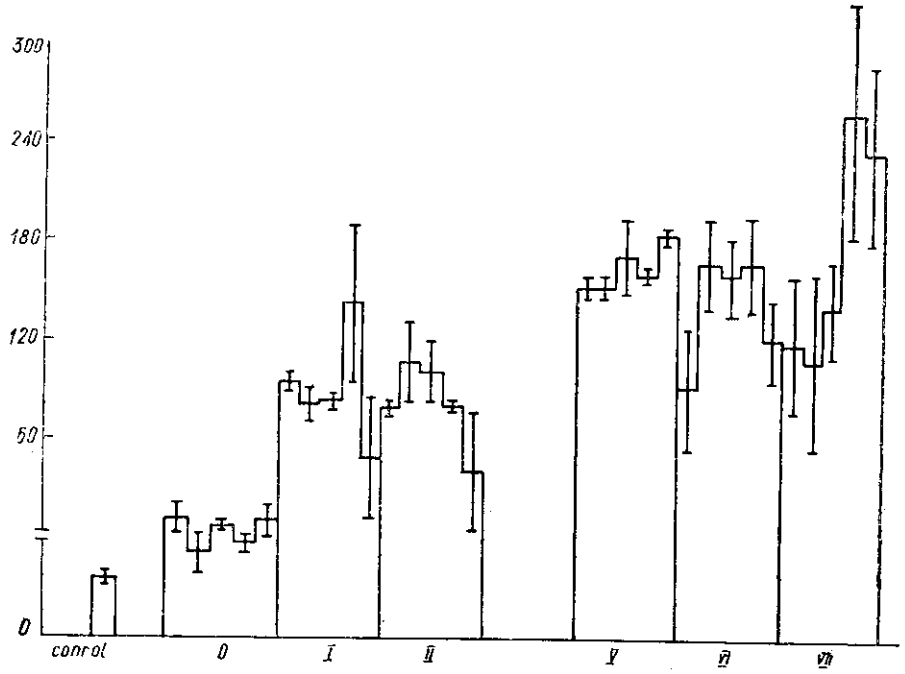


Fig. 13. The content of the protein-product in the samples of the cultural medium under passage of cell subcultures (ordinate axis — ng/ml; abscis axis — passage)

Differences between experimental and control variants are in all cases reliable ($p<0.001$). Comparison of the data from second and seventh passages reveals also reliable differences. There is an impression that the population after cultivation is enriched with cells producing the studied protein in the medium poor in growth factors. If to compare the product of immunoreactive protein in these experiments with the one produced by insulinoma (the data are presented above) it proves that the obtained culture is twice as effective as insulinoma.

After the seventh passage the cells are cloned in the medium with 20 % of embryonal serum [43, 47].

Efficiency of cloning of transfected cells is 1 %. This index is lower in control variants (0.001 %). No clones are observed. Such low cloning efficiency is specified by the fact that cells during several passages grow on half-selective medium and actually are depleted. But the depletion level in the control is so deep that cells do not grow at all after cloning while in the experiment due to the endogenic formation of insulin (or proinsulin) the cloning still proves possible. Five experimental clones are isolated, cells are reproduced, the content of protein-product is determined in samples of the cultural medium. An increased content of insulin-like proteins (60, 80 and 100 ng/ml) is detected among those capable to cloning is likely to be high. To make more exact quantitative analysis a great number of clones must be studied.

Comparison of a total level of synthesis of the product recognized by antibodies to insulin in the culture after some passages under half-selective conditions and clones isolates from this culture as seen from the above data and in fig. 13 shows a large quantitative difference. All clones prove essentially less productive than the initial population. It supports an interesting supposition. If selection at a level of production proceeded under half-selective conditions then the insulin-producing clones would have a selective advantage in cloning. Comparison of the cloning efficiency points to it. But clones (all) isolate the immune-reactive product but in quantities less than initial population from which they are produced. This is possible not for selection aimed at increasing of the product level by cells but for the production proper of the given protein by the cells, i. e. for producing cells on the whole. However in this case one more assumption is needed — a regulatory one. A level of expression under growing conditions in half-selective media is higher than in non-selective ones optimal for cells. A maximal level of expression after cloning (in 20 % serum) returns to its low level corresponding to the one after the first transfection. This suggests that in the residual range of 5′ site of the insulin gene there are sequences that allow regulation of this gene in the nonspecialized cells.

In two experiments where cells transformed by the human insulin gene are selected we use C3HLtk$^-$ cells of a spontaneous tumour of mouse. The cells in this case are implanted with a restriction DNA fragment with the insulin gene. In 10 days after transfection (expression time) cells are held in the medium without serum during 1 month. Cloning efficiency of cells after long-term cultivation in the serum-free medium is less than 0.001 % in the control variant, while after treatment it amounts 2.2 %. Two experimental clones are isolated and reproduced. Insulin-like protein-product in concentration 70 and 100 ng, respectively is found in samples of the cultural medium of these two clones. Dot-hybridization reveals the presence of additional copies of the insulin gene in DNA from cells of these clones. A difference displays itself only under their cultivation in the serum-free medium after transfection. Data on cloning of C3HLtk$^-$ cells after their passage in the growth medium with 10 % serum of cattle may be taken for comparison. Cloning efficiency in the control variant amounts to 13 %, in the experimental variant — 16 % (a difference is not reliable).

Thus the presence and functioning of genome human insulin gene of exogenic origin in fibroblasts of mammals leads to an increase of cell survivability that is expressed under conditions of medium depleted with growth factors (for normal fibroblasts of human and mouse) and medium without growth factors (for tumour cells of mouse). This approach may prove helpful for enrichment of population with cells-transformants and for selection of cells transformed not only by the insulin gene but also by genes of other growth factors. It permits doing without the gene-assistent and a toxic agent. However it brings up the problem on a possibility of the accompanying selection of cells with signs of malignant transformation.

As applied to the problems of gene therapy a key moment here is the maintenance of the normal potential of division in order the cells when cultivated outside the organism do not acquire properties of the malignant phenotype. In this connection we study a possibility of the transformation foci appearance on the monolayer of the transfected cell cultures of LEH and C3H10T1/2. However, transformation foci consisting of morphologically varied and chaotically located cells are not found. Cells C3H10T1/2 transfected by DNA of plasmid *pBR322ins* and initial plasmid *pBR322* are also studied for their tumour forming ability under their subcutaneous administration to new-born irradiated mice of a syngenic line. No tumours are found. Therefore implantation of the recombinant DNA with human insulin gene into normal cells with their subsequent selection on media depleted with growth factors does not lead to

this malignization (at least within the sensitivity of the used methods).

Thus, the experiments on the culture of cells have shown efficiency of the insulin gene expression under constitutive promoters. Now it is necessary to estimate a fundamental possibility of parrying IDDM by implantation of the insulin gene at a level of an experimental animal [48, 49].

After implantation of plasmid *pAins* to animals with artificially induced streptosotocine diabetes one observes in the great majority of mice a considerable decrease of the glycemia with its maximal fall in 6 h after injection. Then a gradual increase of the glucose content takes place but in 10 h its level is low as against control and initial levels. Only in 24 h after DNA implantation the initial indices are set again. A degree of the animal's reaction is different. In some speciments we observe a decrease of the glucose content to a moderate glycemia (7.5-5 mmol/l), in some others — to the norm (4.5-5.0 mmol/l), still in others — to hyperglycemia (2.0-2.5 mmol/l). Some experimental animals died in 6 and 10 h (4 mice). There are also animals with diabetes (9 %) that do not response to the administration of the preparation (tabl. 4).

We perform some control tests to confirm a decrease of the glucose level under the influence of implantation of a plasmid with an active gene human insulin. Under the same conditions we inject empty liposomes and physiological solution into the liver. It proves that opening of the peritoneal cavity before the injection and administration of the physiological solution do not essentially affect the glycemia dynamics. Injection of empty liposomes causes a decrease of the glucose content in the blood of ill animals, but it is less pronounced as against the action of *pAins*. Time is also different: a maximal decrease of the glucose content is observed in 10 h after the injection. There are neither losses of animals nor hyperglycemia in this case.

Thus, the implantation effect of the plasmid carrying an active insulin gene of human is analogous to the action of exogenic insulin preparations injected to ill animals, i. e. one observes a considerable decrease of the glucose content as against control in 6 h after the injection. A high individual variability of the animal's response to the action engaged our attention. It is typical that this variability takes place under injection of pure insulin. A reactive insulin and *C*-peptide in the blood serum of animals are compared after implantation of the recombinant DNA to them during a maximal decrease of glucose (tabl. 5).

The measurements have shown that the content of immunoreactive insulin in animals of control variants is very close (differences are within the measurement error). As for the animals from the experimental group they have more than a 4-fold difference in the quantity of the determined insulin while its absolute quantity under maximal values exceeds 2.5 ti-

Table 4

Glycemia dynamics in mice with artificially induced streptosotocine diabetes after administratio

What is administered	Way of administered	The number of animals
pAins	Into liver	32
pGins	Intraperitoneal	4
Liposomes without DNA	Into liver	10
Physiological solution	Into liver	15
Nothing	(Intact sick animals)	7
Insulin	Intramuscular	33

* $p>0.99$; ** $p>0.999$.

mes a maximal level of control variants. It corresponds to the insulin content in healthy animals. No *C*-peptide is observed in the bloods serum of experimental animals.

As is shown the implantation of the model gene displays a high heterogeneity of animals as to susceptibility to this procedure. There are individuals with a high registered level of β-galactosidase expression in a hepatocyte and there are animals with this index differing from the control but slightly. So variation in the level of the determined insulin in

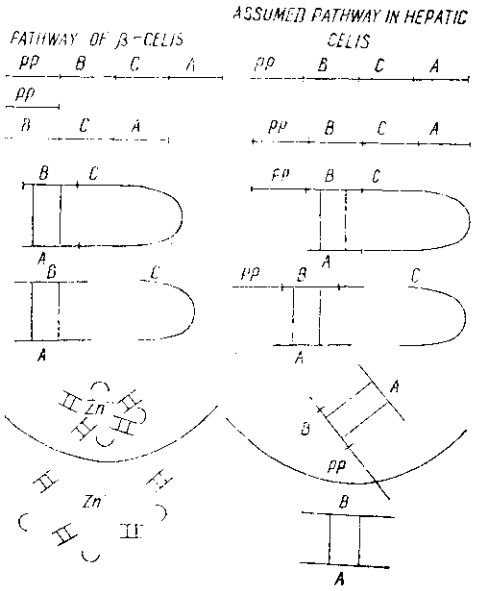


Fig. 14. Ways of processing and transport of preproinsulin and its derivatives (*PP* — prepeptide; *A*, *B*, *C* — peptides)

Table 5

Levels of IRI in 6—8 h after injection of pAins into the liver of rats with artificially induced diabetes

What is injected	The number of animals	Limits of IRI, ME/ml
pAins	4	8.20—34.5
Physiological solution	2	12.8—13.9
Intact sick animals	3	10.3—13.8

the experimental animals proves expected, i. e. it wholly corresponds to the regularities established in the experiments with a marker gene.

The other plasmid *pGins* injected intraperitoneally in the same doses in liposomes also causes a considerable decrease of the glucose level in rats with artificially induced diabetes. Thus, the action of plasmid *pGins* is analogous to the action of *pAins* despite of the differences in the structure of vector molecules and injection methods (table 4).

We study the effect of the recombinant plasmid *pAins* on healthy Wistar rats. An observed decrease of the glucose level in healthy animals is also different. In some animals it is as in the control, in others — falls about twice. It should be noted that in the control group (as well as in the experiments at the moment of injection) there are no essential deviations from the average level. A relatively small decrease of the glucose content in rats susceptible to the administration of the recombinant mo-

of pAins and pGins plasmids

Glucosa, mmol/l				Loss of animals
Time of plasmid administration, h				
0	6	10	24	
9.0—29.6(17.5)	1.7—21.8(10.4)	5.3—25.7(14.6)	7.0—22.0(15.6)	5, Hypoglycemia
19.0—22.7(20.7)	4.3—13.2(9.1)	8.7—14.2(11.4)	13.7—18.0(15.8)	1, Hypoglycemia
12.7—22.5(19.9)	11.0—23.0(17.2)	3.4—20.6**(12.6)	6.2—22.6(16.4)	—
8.9—22.5(16.5)	9.6—23.1(16.0)	5.6—22.9(15.2)	7.8—19.7(14.8)	—
9.0—22.4(17.5)	8.6—21.6(15.7)	10.6—22.8(17.8)	6.8—21.6(17.5)	—
9.0—22.0(16.1)	4.4—16.7*(11.6)	—	—	—

lecules may be attributed to the powerful system of regulation of the glucose content in the blood of healthy animals (table 6).

Analysing the obtained results one should consider also possible nonspecific reaction of the organism. It is known that the largest populations of macrophages (called also Kupffer cells of the liver) are located in the liver and in the peritoneal cavity. They are the first link of the protective system in the mammals organism. These cells populations are heterogeneous as to their functional specialization – a set of receptors, stages of differentiation etc. Liposomes are easily and quickly phagocytized by macrophages. A high activity of the penthosephosphate path for glucose consumption is typical for activated macrophages [50, 51].

It is not the main way of glucose metabolism in the mammals. Its quantity depends on the functional state and may be rather high when the cell transform all its biological activity on biosynthesis and isolation of purposeful products: mediators, enzymes. Glucose consumption by the Kupffer cells increases in this case 3-4 times. It affects this way or the other a total content of glucose in blood. Considering a total quantity of the Kupffer cells, a level of their activation, dynamics of glucose consumption, its total initial level, a total quantity of glucose at the beginning of the experiment and in its peak activity and time necessary for it, difference in time with an action of insulin (a small lag necessary for penetration and expression start of the gene), variations in animals susceptibility to insulin and the implanted gene etc., a nonspecific action of the glucose absorption well accounts for the action of empty liposomes. An effect of the administered insulin gene of human is well pronounced against the background of this nonspecific decrease of the glucose level in the control.

Coincidence of the glucose absorption dynamics under administration of pure insulin gene into the liver of ill animals allows a supposition that transitory transformed hepatocytes isolate not proinsulin but insulin [49]. Since for hepatocytes a correct level of proinsulin processing and its transport from cells prove incredible we assume that a succession of stages for insulin maturation is changed. It is as follows: after proinsulin synthesis there proceeds a correct laying, then *C*-peptide is splitted off and finally the form not at all formed in β-cells (tentatively it may be called preinsulin: correctly set *A*-peptide chains without *C*-peptide but with prepeptide) is isolated due to prepeptide through an internal cell membrane where it is removed while a functionally active insulin comes into the intercellular space [1]. Under this transport *C*-peptide does not at all leave the cell (fig. 14).

Expression peculiarities of the insulin gene implanted into the cells of the culture as well as cloning of these cells and a level of the tested product after the expression underlies a supposition that the fragment of the genome under consideration contains certain regulatory sequences which allow for the regulation in the nonspecialized cells. Fig. 15 presents

Table 6

Administration of plasmid pAins and pGins to healthy animals

What is administration	Way of administration	The number of animals
pAins	Into liver	11
»—»	Intraperitoneal	5
pGins	Intraperitoneal	5
Liposomes	Into liver	5
Physiological solution	Intraperitoneal	3
Nothing	(Intact sick animals)	6

a primary sequence of the insulin gene and adjacent regions from 5′ and 3′ end. The cloned fragment under consideration (in all above experiments) is located between *BglII* and *TaqI* sites (sequences 2017-2022 and 4819-4824).

Comparison of the thoroughly studied structure of the regulatory sites of insulin I rat's gene shows that a sequence in position 217-197 critical for tissue-specificity is absent in our constructions (*BglII* site begins from the nucleotide in position 167).

Another negatively (for others except β-cells) controlling element is between 100 and 91 nucleotides. It is present in our constructions. However due to sequence 110-100 that specifies binding of a tissue-specific factor starting the transcription the expression of this fragment containing the insulin gene takes place in the cells that do not synthesize insulin in the norm [52, 53]. Canonical promoter sequences TATAAA-box (nucleotides in position 28-24) are also preserved in this fragment. CAP-site (*o*-position) is not disturbed as well. It allows varying a level of expression in different types of cells. It is very significant that such residual regulatory elements may essentially affect the expression in cells of different individuals in whose organism a level of synthesis of certain proteins or their affinity to the given sites are somewhat changed. In the norm insulin is synthesized only in β-cells of the pancrease (and in some nerve ones). Therefore the insulin gene sites recognized by the tissue-specific factors in transcription of other cells are intended not for it. They are simply close to the insulin sites because of an accidental coincidence (a sequence is very short).

Other not yet found regulatory sequences affecting the expression in the specialized cells are also possible. In this case our attention is engaged by an exceedingly high saturation of 5′ nontranslated region G — C with nucleotide pairs (G in significant filament). They amount to 49.7 % in sequence 885-1044, 60.9 % — in sequence 1380-1816. There is an abundant region G — C — in 3′ they amount to 51.5 % in position 4011-4079. Such cluster deviation can hardly be caused by a random distribution in 25 %. These regions from 5′ end and from 3′ end are nontranslated. Their role in the regulation suggests itself. These G — C regions from 5′ end are taken away from the fragment under analysis while from 3′ end — are kept. A kind of asymmetry appears there. Possibly it contributes into the specificity of the described expression.

Substantiation of a fundamental possibility of gene therapy of atherosclerosis. Atherosclerosis is taken by us as the second example of mass pathology to be treated by the methods of gene therapy. If in case of IDDM a choice of the gene with en expected therapeutic effect is evident then in case of atherosclerosis at first glance it seems to be impossible. Atherosclerosis is a multifactor pathology [54] with many different physiological, biochemical and molecular processes contributed into it.

Really one cannot expect the only one universal means for all cases. However medical experience testifies that among all the various reactions

Glucose, mmol/l			
Time after plasmid administration, h			
0	6	10	24
4.0—5.1 (4.6)	2.7—4.3 (3.5)	3.3—4.6 (4.0)	4.0—5.7 (4.8)
4.2—6.0 (5.0)	2.0—5.7 (3.8)	2.3—5.4 (3.6)	3.1—5.9 (4.8)
4.5—6.0 (5.1)	2.7—5.0 (3.8)	3.1—5.6 (4.3)	4.2—5.7 (5.0)
4.3—5.5 (6.0)	2.4—5.3 (3.7)	3.9—4.9 (4.5)	3.9—5.7 (4.7)
4.4—5.1 (4.8)	3.9—4.7 (4.4)	4.1—5.4 (4.8)	4.3—5.1 (4.8)
4.3—5.7 (5.0)	4.3—5.7 (5.2)	4.0—5.1 (4.9)	3.5—5.6 (4.7)

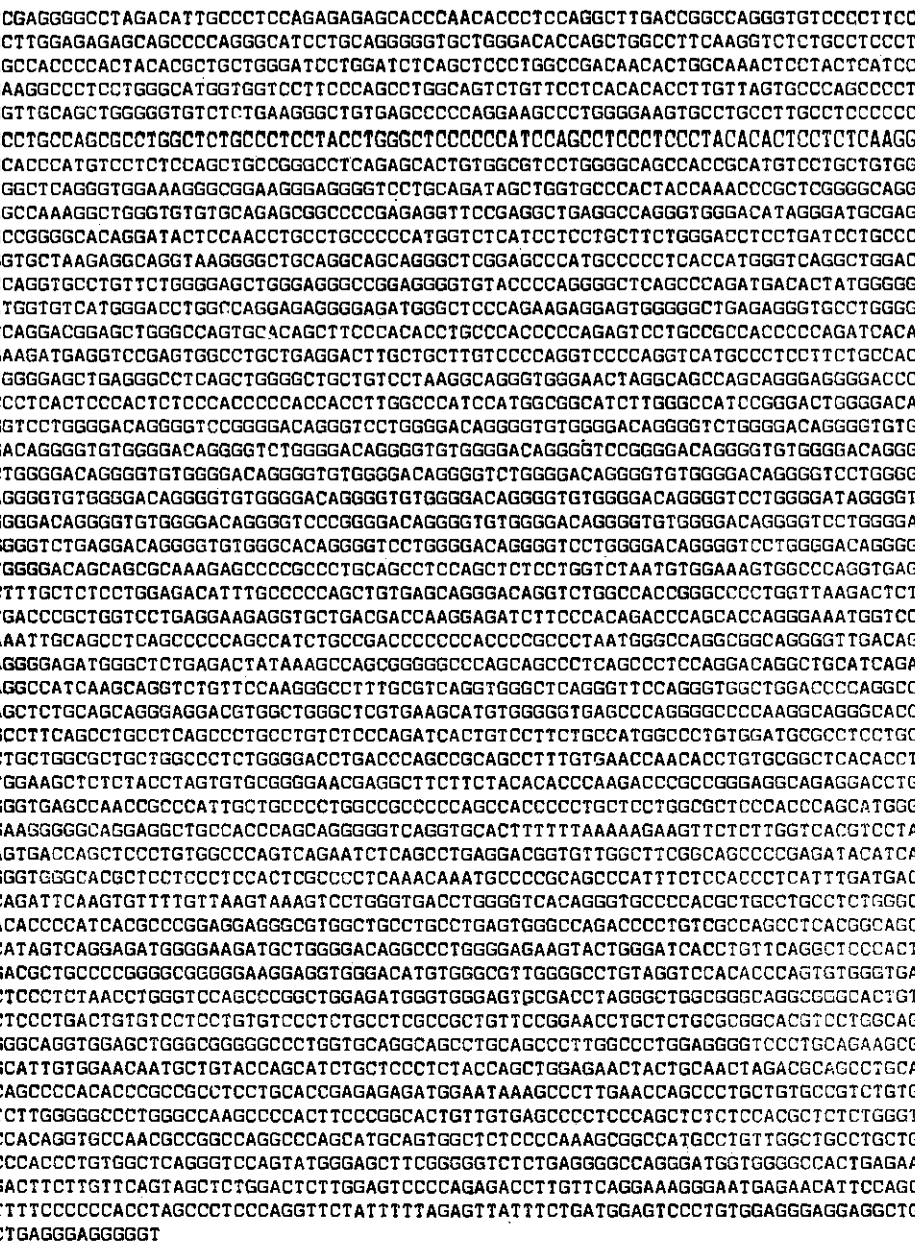


Fig. 15. Primary sequence of insulin gene and adjacent region from 5′ and 3′ end

participating in the development of atherosclerosis there is a certain leading motive.

In compliance with modern notions on a significance of lipid and lipoproteid metabolism in development of atherosclerosis numerous clinical and experimental measures on prophylaxis and treatment of this disease are based on using means that decrease the lipid content in blood [55]. Alongside it is known that development of atherosclerosis is connected not only with changes of lipid but also protein part of lipoproteids — apoproteins. Apolipoprotein *AI* is the main component (70 %) in the protein fraction of high-density lipoproteids (HDLP) which can regulate a level of cholesterin in blood and remove it from cells of vessels. A significant antiatherosclerogenic role of this fraction in lipoproteids is confirmed by numerous epidemiological studies showing that human individuals with a high level of HDLP do not suffer atherosclerosis while

shortage of this fraction of lipoproteids correlate with a risk of cardiovascular pathologies. It determines new approaches to searching of antiatherosclerotic preparations among substances affecting HDLP synthesis.

Proceeding from the above it seems promising in cases of increased level of cholesterin, changes in correlations of the lipoproteid fractions

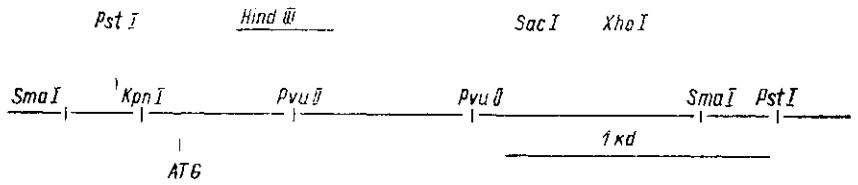


Fig. 16. Restriction map of the human gene *apoA1*

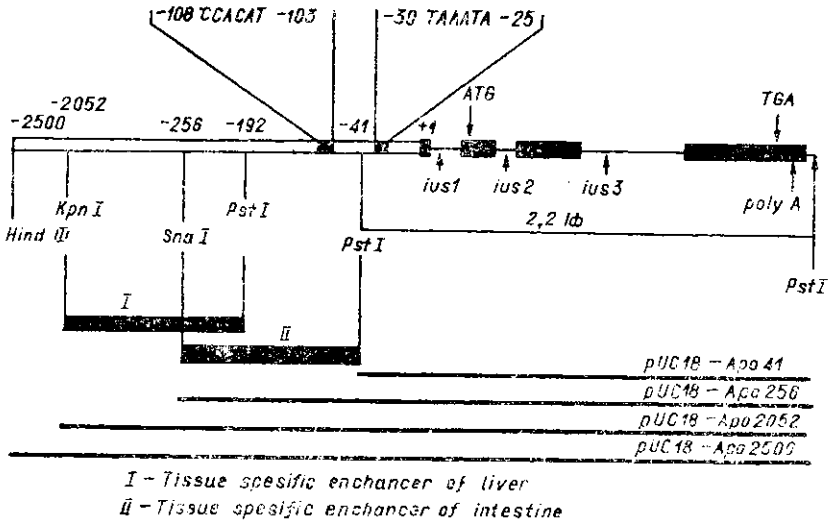


Fig. 17. Structure of the human gene *apoA1*

and other the like disturbances of lipid metabolism envolving development of atherosclerosis to increases HDLP level by genotherapeutic correction using a cloned human gene *apoA1*.

Clone with positive reproducible signals under its probe hybridization to human gene *apoA1* is obtained after screening of about $3 \cdot 10^5$ recombinant phages from the library of human genes. DNA of the recombinant phage is produced in preparative quantities with its splitting by different restrictases and subsequent Sauthern blot-hybridization of DNA fragments separated by electrophoresis.

PstI fragment (about 2.2 kb) of human DNA is subcloned in composition of a bacterial vector *pUC18* in two orientations (plasmid *pUC18apo* and *pUC18apo'*). Mapping of the cloned human DNA fragment using restriction endonucleases *HindIII*, *PvuII*, *SacII*, *KpnI*, *XhoI*, *SmaI*, *TaqI* confirms identity in the length of the obtained restrictions fragments with those of human gene *apoA1* whose restriction map is shown in fig. 16.

A primary structure of *PstI-HindIII* fragment of DNA from 5′ end of the cloned gene is determined. No changes are observed in the nucleotide composition as against previously described for human gene *apoA1* [56]. The presence of seven-member AT-rich sequence (ATAAATA) which is a part in the promoter region in the natural gene surrounding is confirmed in the cloned DNA fragment in position — 264-258 in relation to ATG codon. Scheme of human gene *apoA1* and sequences specifying its regulation are shown in fig. 17. As we see *PstI* fragment contains incomplete promoter but some elements responsible for tissue-specificity are

absent. So, when constructing DNA we proceed from the necessity to introduce additional regulatory elements into its composition.

It is known from literature that *alu*-repetitions in human contain DNA sequences that may act as enhancer for RNA-polymerase II and as promoter for RNA-polymerase III [57]. Therefore to study the expression of the cloned gene we construct a recombinant plasmid *pAL1apo* which is different from *pUC18apo* by the presence of the polylinker from one human *alu*-repetition that is cloned by *BamHI* site and located in the 5′ end of the gene.

Recombinant plasmids are used to transform mice fibroblasts of Ltk⁻ line which in 24 h are washed and poured with a serum-free medium but

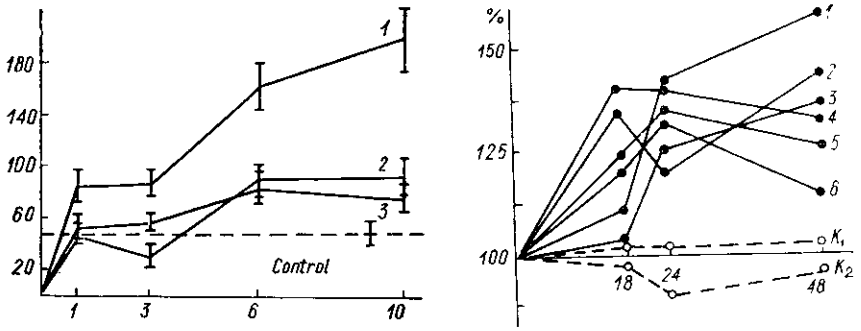


Fig. 18. The content of human *apoA1* in the culture medium after transfection of mice fibroblasts by different plasmids: *1* — *pAL1* *2* — *pUC18apo′*; *3* — *pUC18apo* (ordinate axis — protein, ng/ml; abscise axis — days)

Fig. 19. Change of ChS-HDLP after administration of human *apoA1* to adult rabbits

with 2 % glucose. In some periods of time this glucose is replaced for fresh portions to perform immunoenzymic testing of human *apoA1* in samples.

At each stage we obtain lisates from cells for parallel testing of protein in them. The results of testing are shown in fig. 18.

No protein *A1* is found in cell lisates of human that evidences for its prevailing secretion into the cultural medium. A maximal protein secretion into the cultural medium (under conditions of our experiments) is observed in 3-6 and 6-10 days after administration of plasmid DNA. In all cases during 10 days one observes a reliable excess of the quantity of the tested protein over the control (cells without implanted plasmid DNA and cells with the implanted *pUC18*) after transfection of *pAL1apo*. The quantity of the tested *apoA1* after transfection of *pUC18apo* and *pUC18apo′* (different gene orientation) reliably exceeds the control background on 3-6 and 6-10 days. However its quantity is much lower than in the variant with *pAL1apo*. As it is expected an expression level of the gene in the cell culture does not depend on its orientation in the bacterial vector and is everywhere low if to subtract the control background. It may be attributed to the fact that not all the sequences of the promoter site are present in the cloned fragment of human DNA. Introduction of one human *alu*-repetition into the recombinant molecular enhances the gene expression. To our mind, it happens due to restoration of the enhancing function of a tissue-specific character. It also demonstrates that *alu*-like scattered repetitions possess important properties worthy of attention. On the one hand they contain sites capable to enhance expression of the genetic material. On the other hand, their use because of their high occurence in the human genome may increase a frequency of homologic recombination, thus promoting integration of foreign genetic material. These properties make *alu*-repetitions very promising under construction of vectors.

The next stage is devoted to study of the expression of human gene *A1* under its administration into experimental animals. For the first ti-

me the problem of gene therapy of age pathology is stated and experimentally solved.

Human gene *apoA1* in plasmid *pAL1apo* is implanted to experimental animals (adult and old rabbits). It is shown that irrespective of the animal's age one observes human protein *apoA1* in their blood. It is detected by immunoelectrophoresis in the agarose gel with a specific antiserum to this protein. Immunoprecipitation does not take place in the blood of control animals. The quantitative estimation of human *apoA1* detected in the blood plasma of rats (under standard human serum) demonstrates that its content in 24 h after gene implantation varies from 0.5 to 0.25 mg/length in old rabbits and to 0.965±0.30 mg/length in adults.

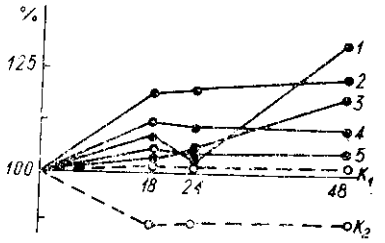


Fig. 20. Change of ChS-HDLP after administration of human *apoA1* to old rabbits

Changes in the content of Chs-HDLP are observed in blood of experimental rabbits in 18, 24 and 48 h. From data in fig. 19 we see that they differ in different animals. However one observes mainly a growth of Chs level of LP fraction.

As early as in 18 h we observe a pronounced change of Chs-HDLP content in experimental animals of both age groups. Changes in adults are more pronounced than in old animals. However it is pronounced enough in the old specimens too. The like changes we observe in total cholesterin of the blood plasma in rabbits [55]. Such regularity is observed also in a change of a per cent content of HDLP as against other fractions of LP (table 7) in adult animals. Changes in old animals are observed only in 48 h. Analysing the data it is necessary to take into account a well-pronounced individual response of animals to the gene implantation (fig. 19 and 20). So, the average induces do not reflect the situation on the whole.

Changes of relative Chs-HDLP concentrations and HDLP fraction are within diurnal variations of these parameters in control animals with administered empty liposomes (K_1, K_2). Data on the gene expression in a cell culture do not always adequately reflect the processes proceeding at a level of intact organism. Consequently the expression of human gene *apoA1* is very important under its direct implantation into animal's cells.

Table 7

The content of different fractions of lipoproteids in blood of adult and old rabbits after implantation of human apoA1 gene (relative concentration, %)

Conditions of the experiment	Adult			Old		
	LDLP	VLDLP	HDLP	LDLP	VLDLP	HDLP
Control (administration of liposomes without genetic material)						
Mitial level	35.4±1.5	24.6±1.6	40.0±3.2	51.4±3.5	19.6±2.1	29.0±1.8
18	34.6±1.7	25.0±1.4	41.4±2.9	50.7±4.1	21.0±2.4	28.3±1.6
24	35.1±2.1	24.7±1.8	40.2±2.5	51.1±3.7	19.8±2.1	29.1±1.4
48	36.0±1.8	25.8±1.5			20.7±2.4	29.0±1.3
Gene implantation						
Mitial level	33.2±1.8	26.7±1.9	40.1±2.8	52.3±2.0	21.0±1.6	26.7±2.1
18	33.5±1.2	20.0±1.5	46.5±2.3	53.0±3.4	22.1±3.4	4.29±2.1
24	33.6±1.7	19.1±1.3	47.3±2.1	51.6±3.1	23.0±1.4	25.4±2.7
48	32.7±1.3	22.3±1.2	45.0±2.3	47.4±3.1	20.0±1.6	32.6±2.1

Note. 18, 24, 48 — time in h after gene implantation.

A level of expression depends on the peculiarities of the construction of recombinant DNA containing human gene *apoA1* (*PstI* fragment) and additional regulatory elements. So, we cannot show the expression of gene in plasmid *pUC18apo* and *pUC18apo'* because of either a very low level of the gene expression with a fragment of its own promoter into the composition of molecular structures (below the sensitivity of the detection method) or impossibility of the expression due to tissue-specificity of its regulation that is more probable. A fragment of 5′ nontranslated region of human gene *apoA1* (—256-41, fig. 17) performing a function of a liver enhancer in the natural nucleotide surrounding of the gene is absent in composition of molecular construction. It is supported by the fact that implantation of human gene *apoA1* in the molecular construction with human *alu*-repetition into the animals results in the easily-tested human protein in the blood of experimental animals though these constructions slightly differ by a level of the gene expression in the culture. Mechanisms of changes in certain physiological parameters in the blood of model animals (Chs-HDLP, HDLP) registered in this case need further investigation. However comparison of the absolute values of these deviations with a corresponding level in control animals (liposomes without implanted gene) permits a supposition that it is connected with an expression of the implanted human gene *apoA1*. Data obtained after implantation of DNA *pUC18apo* and *pUC18apo'* to the rabbits support the above supposition, i. e. implantation of the gene in the construction that does not provide a certain level of its expression does not lead to changes of lipid characteristics in the organism. Heterogeneous changes observed in the organism of animals during injection of expressing vector molecules may be attributed to individual variability of the animals to the given gene as well as in previous experiments with other genes.

The obtained data on the expression of human gene *apoA1* at a level of intact model animals show that a physiological effect of the protein secretion after the gene implantation is short-term with subsequent damping. Therefore long-term experiments to simulate physiological processes proceeding in the organism require fundamentally new approaches for construction of the vector molecules. As is known, implantation of the given gene in the retrovector is a usual methodical solution. However retrovectors cause certain misgivings. The use of eukaryotic ARS-sequence of DNA may be an alternative, to our mind.

To verify a possibility of this approach in principle we subclone previously cloned *EcoRI* fragment of maize genome DNA that possesses ARS-function in *S. cerevisiae* [59] and *N. tabacum* [60]. The recombinant plasmid is entitled *pAAA* (*apo-alu*-ARS). ARS is taken from the plant that is evolutionary very far from mammals with the aim to avoid, if possible, recombination events because of homology.

Ability of this molecular construction to be preserved in the cell culture is estimated by determining transformation frequency of competent *E. coli* cells of plasmid DNA isolated from cell culture in certain periods of time after transfection (table 8) and by hybridization analysis of the same plasmid DNA. DNA *pUC18* or human gene *apoA1* are used as a probe. Analogous operations but without implantation of plasmid DNA are performed with control cells.

The obtained data testify that plasmid *pAAA* with maize ARS-sequence in a series of recombinant molecules containing human gene *apoA1* is best of all preserved in the cell culture. In this case we observe a smooth descending of the number of *E. coli* transformation. If to suppose that this phenomenon is resulted from the replication of plasmid DNA in the cell culture then a decrease of the number of transformants in time may be attributed to a negative balance of the replication process and biodegradation of the implanted genetic material. A character of changes in the X-ray film exposure intensity under hybridization analysis is analogous to the data obtained by comparison of transformation frequencies of different plasmids isolated after transfection of mouse fibroblasts.

Data obtained after passage of transfected mouse fibroblasts on serum-free medium may serve as an indirect substantiation of at least ability of DNA *pAAA* to preserve better after Ltk⁻ transfection. Their ability to cultivation decreases in the order the plasmids are presented in fig. 9, i. e. cells with implanted *pUC18apo* (weakly secreting *apoA1*) die faster than the cells with implanted *pAL1apo* while these in their turn die faster than those with implanted DNA *pAAA*. We may suppose that since *apoA1* is a cofactor of the enzyme lecitin: cholesterin: acyltransferase that is significant for cholesterin metabolism in a cell then under conditions of deficiency (serum-free diet) the ability to synthesize *apoA1* becomes for a cell a critical factor determining its survivability. Therefore those cells that synthesize more *apoA1* survive in the first turn, i. e. the cells with implanted DNA *pAAA* containing ARS sequence of DNA.

It is well known that different kinds of laboratory animals differ in their susceptibility to atherosclerosis. Rabbits belong to the group with easily induced atherosclerotic pathology. In contrast to them there are animals which practically do not suffer this disease. In this connection it appears interesting to study the effect of human gene *apoA1* implantation on certain parameters of blood serum in rats as representatives of the population resistant to this disease [61].

Preliminary experiments have shown that implantation of human gene *apoA1* in plasmid *pAL1apo* and *pAAA* makes it possible to test human protein *A1* reliably in 48 h in rat's blood plasma only when using recombinant DNA containing ARS. Since there are no data testifying to a probable replication of *pAAA* we suggest analogously with the cell culture that the given molecular construction is better preserved when implanted to the animals, so we choose it for our further work. Blood for tests is taken on 2nd and 7th day after implantation of human gene *apoA1*. The experiment is performed with 36 adult and 39 old animals.

DNA *pAAA* in a liposome is administered into the liver of adult and old rats. On the 2nd and 7th day the human protein *apoA1* is tested in animals blood with simultaneous determination of certain lipid parameters. Protein is detected on the second day. Its quantity in adults is 8-10, in old animals — 4-6 μg/ml. In seven days after gene implantation we do not observe human protein *apoA1* in the blood of both adult and old animals. It is attributed to the fact that either ARS-sequence of DNA in a molecular construction is not effective enough for the given system of cells or irrespective of the plasmid better preserved in the organism there proceeds a transitory expression of human gene *apoA1* whose level (considering protein clearance) does not allow testing *apoA1* in 7 days. The appearance of human *apoA1* in the blood serum is accompanied by a change of concentrations of different types of LP, particularly HDLP, correlations of $HDLP_2$ and $HDLP_3$ subclasses (table 9).

Table 8

A level of E. coli transformation by plasmids isolated from transfected mouse fibroblasts of Ltk⁻ line

Plasmid	Transformation frequency of E. coli per 1 μg of plasmid DNA	Transformation frequency of *E. coli* plasmid DNA after transfection of fibroblasts	
		In 3 days	In 5 days
pAAA	$(1.4\pm0.2)\cdot10^5$	651±19	455±18
pAL1apo	$(2.1\pm0.3)\cdot10^5$	215±14	55±50
pUC18apo'	$(3.0\pm0.3)\cdot10^5$	230±16	68±80
pUC18	$(4.0\pm0.4)\cdot10^5$	280±17	73±90
Control	—	—	—

Note. For transfection of Ltk⁻ we take 20 μg of plasmid DNA for one bottle of cells (about $5\cdot10^6$ of cells). The quantity of grown *E. coli* transformants are presented in terms of all genetic material isolated from fibroblasts.

On the second day after implantation of the cloned human gene *apoA1* one traces a tendency of the HDLP content increase in the blood serum of rats especially noticeable in old animals. The quantity of HDLP increases in adult animals by 20 %, in old ones — by 30 %. Since HDLP as well as other LP is determined by the protein quantity, so the obtained data support a supposition that HDLP increases both at the account of human *apoA1* formation and by stimulation of rat's own HDLP synthesis due to implantation of human gene *apoA1*. A considerable increase of VHDLP in adults (by 36 %) and especially in old animals (by 52 %) accounts in favour of the supposition on the effect of human gene *apoA1* expression on LP synthesis in rat's liver.

Implantation of *apoA1* gene leads not only to a total growth of HDLP in the blood of animals but also to a change in the content and correlations of subclasses of the LP-$HDLP_3$ and $HDLP_2$, these changes being different in old and adult animals. Implantation of gene *apoA1* into the rat's liver causes a considerable increase of $HDLP_2$ in old animals only (by 44 %), while the content of $HDLP_3$ grows almost similarly both in old and adult rats (by 26 % and 24 %, respectively). As a result $HDLP_2/HDLP_3$ ratio increases in old rats while in adult ones it adult ones it falls (by 13.8 %).

As is known $HDLP_3$ is able to accept Chs from periphery cells and to transform into $HDLP_2$ in this case. $HDLP_2$ in its turn conveys the excess of Chs into the liver cells. Different changes in the content and subfraction of HDLP under conditions of human gene *apoA1* implantation both in adult and old animals testify to the age changes in cholesterin-acceptor function of HDLP in the old age.

Implantation of human *apoA1* into rats changes not only the quantity of HDLP but also their protein composition [62]. Under conditions of gene implantation one observes in adult and old animals a noticeable tendency to an increase (by 18.2 and 30 %, respectively) of a relative content in a subclass of *apoA1* in $HDLP_2$ (table 10). An increase in the content of this subfraction is observed also in $HDLP_3$ but only in old animals.

Shifts in the protein composition of HDLP isolated from rat's blood are accompanied by the pronounced changes in their lipid composition (table 11). The content of phospholipids and free Chs in HDLP isolated from the serum of old animals increases by 34 % and 69.5 %, respectively. In this case we observe a distinct tendency to a decrease of Chs esters content (by 18.4 %). However a total content of Chs-HDLP is not changed which testifies that there is no enhancement of the additional synthesis of Chs under implantation of gene *apoA1*.

Changes in lipid composition of HDLP under conditions of gene implantation cause shifts of corresponding indices in subclasses of $HDLP_2$ and $HDLP_3$ (table 11). In old rats under the human gene *apoA1* implantation in $HDLP_2$ one observes a considerable growth (by 67.3 %) of the

Table 9

Effect of human gene apoA1 implantation in a liver of intact rats on concentration of fraction of lipoproteids and subclasses of HDLP in blood serum of different age rats

Lipoproteids, mg of protein per 1 ml of blood	6—8 months		26—28 months	
	Control	Experiment	Control	Experiment
HDLP	0.152±0.040	0.616±0.024**	0.517±0.026	0.672±0.032**
$HDLP_2$	0.116±0.004	0.124±0.006	0.124±0.007	0.179±0.009**
$HDLP_3$	0.397±0.035	0.491±0.020**	0.393±0.020	0.494±0.033**
LDLP	0.073±0.002	0.082±0.005	0.079±0.006	0.093±0.010
VLDLP	0.073±0.006	0.099±0.004**	0.102±0.010*	0.155±0.030

* Statistically significant differences between adult and old animals; ** differences between control and experiment ($p<0.05$—0.01).

content of phospholipids. A correlation between Chs fractions changes in favour of free Chs (its quantity increases by 29 % while the content of Chs esters decreases by 21.4 %). As a result an FChs/EChs ratio grows considerably in old rats (by 60 %). Taking into account a considerable increase of the protein mass of $HDLP_3$ one may assume that the outflow of Chs from cells in $HDLP_2$ less enriched with Chs esters is enhanced in old rats under conditions of gene implantation.

The obtained data testify that the implantation of human gene *apoA1* into rat's liver leads not only to an increase of HDLP in the blood of tested animals (hyper-alfa-lipoproteidemy) in correlation of their subclasses but also to a change of their protein and lipid composition. The revealed shifts are more pronounced in old animals. This result proves most unexpected. To give it an adequate interpretation one needs additional studies. We may suppose as yet that the response of animals to the implanted key gene controlling the given processes differs greatly. Probably study to the molecular fundamentals specifying such differences may have an independent significance in the measures aimed at treating this disease. It should be noted that implantation of human gene *apoA1* to experimental animals along with changes registered in the blood causes essential events in cells of other organs and tissues. One observes growth in the content and activity of cytochrome P-450 [63]. Its quantity grows more considerably and intensively in adult animals than in old ones (by 54 and 46 %, respectively).

It may testify in favour of the induction of the expression of genes in the monooxygenase system under these conditions. However it is not inconseivable that such changes in the intensity of macrosomal oxidation under conditions of gene implantation may be a reaction on an increase of the concentration of Chs coming into the liver by means of HDLP.

Therefore implantation of human gene *apoA1* to rats results in the expression on the implanted gene and the appearance of human *apoA1* in the blood of experimental animals. It is accompanied by an increase of HDLP level expecially in the blood of old rats and of the content of human *apoA1* in them. In old animals the quantity of *apoA1* increase at

Table 10

Effect of human gene apoA1 implantation into the liver of intact rats on a protein spectrum of high-density lipoproteids in rats of different age

Protein	m. m.·10³	Relation to the total optical density, %			
		6—8 months		26—28 months	
		$HDLP_2$	$HDLP_3$	$HDLP_2$	$HDLP_3$
apoC	12.5	—	—	—	—
c		1	3	1	3
e		1	17.6±1.3	1	3
apoA1					
c		58.2±3.0	36.1±4.7	20.0±2.2	14.0±1.2
e		68.8±4.5	22.3±4.8	26.1±2.4	21.0±2.3
Arginin-rich protein	35				
c		25.3±3.0*	10.0±2.3	40.8±4.8	23.5±3.2
e		15.0±3.2	23.4±5.7	50.3±6.1	29.1±3.3
apoA1IV	51				
c		8.8±0.6	15.9±2.0	30.1±2.2	13.4±2.1
e		8.1±1.2	13.3±0.5	17.8±1.2*	15.2±2.5
apoA1	2				
c		6.1±1.6	19.0±4.3	8.0±2.7	42.2±2.5
e		8.3±1.9	20.0±4.9	9.3±1.2	25.1±4.5*
High-molecular proteins	80				
c		2	16.1±3.3	1	6.4±1.1
e		1	13.3±3.8	5.1±1.0	7.2±1.3

* Statistically significant differences in the experiment (e) as against control (c) ($p<0.05$).

the account of its growth in the subfraction of $HDLP_3$. Other indices also change.

Summing up the data of this section one should distinguish the following principle moments in the whole complex of shifts after implantation of human cloned gene *apoA1* to experimental animals. First, these are changes connected with synthesis of human *apoA1* gene and its appearance in the organism of experimental animals. Second, it is a complex of gene regulatory shifts (growth of VLDLP content, change in the correlation of apoproteins, change of the qualitative composition of LP). Third, a generealized response of the cell (induction of the macrosomal oxidation). Attention is engaged by the fact that synthesis of human *apoA1* in the organism of rats is more pronounced in adult animals while gene-regulation shifts — mainly in old ones. Apparently it is connected with the fact that the genome regulation in the ageing changes itself being a fundamental link in the ageing mechanisms [61]. Potentiality of gene therapy to affect the ageing opens qualitatively new prospects for treatment of what seems impossible — therapy of age pathologies. It appears interesting that implanting of a present gene for a corresponding system that ensures expression makes it possible to obtain the effect of enhancement of the processes which themselves do not promote development of atherosclerosis in the given organism (rat). Such enhancement leads, to our mind, to elimination of cholesterin from a cell membrane, thus becoming more important as was previously assumed.

Thus implantation of gene *A1* has resulted in the expected physiological effect which is expressed in an increase of *A1* protein and in all subsequent changes that are the consequence of such increase. Now it is necessary to find out a fundamental possibility of therapeutic effect under the action of implanted gene *A1*. With this aim we use a commonly accepted model of cholesterinemy in rabbits caused by feeding them with food rich in cholesterin. After cholesterin load one observes a sharp increase of the cholesterin content in the blood plasma of rabbits which then slowly decreases. Simultaneously the content of cholesterin grows due to high-density lipoproteids.

Table 11

Effect of human gene apoA1 implantation into the liver of intact rats on lipid composition of HDLP and their subclasses in the blood serum of rats of different age

Lipids, mmol/mg of protein	HDLP		$HDLP_3$		$HDLP_2$	
	Control	Experiment	Control	Experiment	Control	Experiment
			6—8 months			
Phl	0.97±0.08	0.82±0.06	1.03±0.10	0.45±0.05**	1.52±0.11	1.61±0.10
TChs	2.84±0.09	2.87±0.14	1.94±0.18	2.05±0.21	3.51±0.06	3.79±0.36
FChs	0.58±0.03	0.50±0.039	0.30±0.04	0.23±0.02	0.53±0.03	0.33±0.04**
EChs	2.26±0.12	2.35±0.09	2.10±0.19	1.82±0.20	2.90±0.19	3.08±0.32
Phl/TChs	0.34±0.02	0.29±0.01	0.30±0.01	0.22±0.03	0.43±0.02	0.47±0.04
Phl/FChs	1.95±0.14	1.86±0.21	2.40±0.30	1.92±0.34	2.87±0.35	4.83±0.49**
FChs/EChs	0.26±0.02	0.21±0.02	0.14±0.02	0.13±0.02	0.18±0.02	0.11±0.03
			26—28 months			
Phl	0.63±0.04*	0.85±0.08**	0.90±0.09	0.56±0.07**	0.68±0.13*	1.13±0.15**
TChs	3.45±0.20*	3.22±0.14	2.58±0.29	2.78±0.30	4.91±0.51	3.94±0.40
FChs	0.46±0.06	0.78±0.06**	0.36±0.05	0.40±0.03	0.42±0.05	0.55±0.03
EChs	2.99±0.26	2.44±0.14	2.65±0.27*	2.37±0.28	4.25±0.54*	3.38±0.39**
Phl/TChs	0.18±0.02*	2.26±0.01**	0.43±0.08	0.20±0.03**	0.22±0.05*	0.20±0.04
Phl/FChs	1.59±0.10*	1.62±0.21	2.30±0.25	1.49±0.16**	1.62±0.35*	2.05±0.29
FChs/EChs	0.15±0.02	0.32±0.03**	0.15±0.02	0.17±0.01	0.10±0.01	0.16±0.01

* Statistically significant differences between adult and old animals; ** differences between control and experiment ($p<0.05$—0.01); Phl — phospholipids; TChs — total cholesterin; FChs — free cholesterin; EChs — esterified cholesterin.

Fall of a total cholesterin is accompanied by a fall of the HDLP cholesterin content (fig. 21).

Implantation of gene *A1* essentially changes the situation. A total content of cholesterin in blood in a day after the end of the cholesterin load proves much lower than in the control (i. e. in the variant with cholesterin load but without implanted gene *A1*). At the same time the

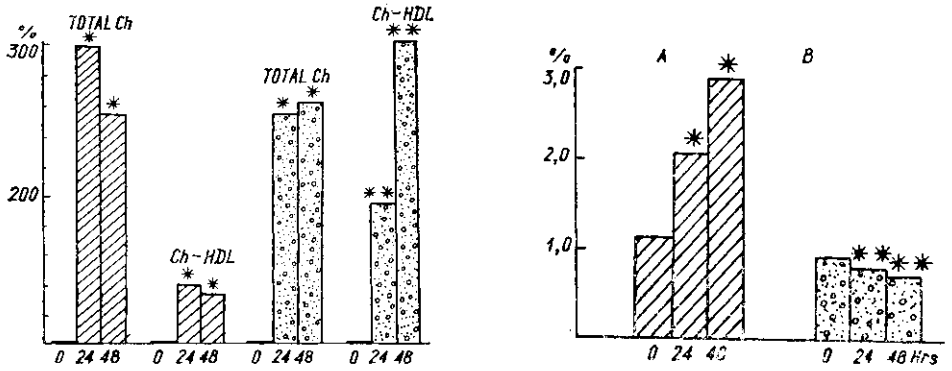


Fig. 21. Effect of transformed human gene *apoA1* on development of hypercholesterinemy in rabbits (there and on the fig. 22 * — statistically significant compared to the initial level; ** — the same to cholesterol load)

Fig. 22. Effect of transformed human gene *apoA1* on atherogeny coefficient in rabbits: *A* — cholesterol load; *B* — the same after transfer of human *apoA1* gene)

content of cholesterin in HDLP increases several times. Moreover it continues growing on the second day in contrast to the control when it falls (fig. 21).

Atherogenic coefficient changes correspondingly: it grows in the control and falls in the experiment (fig. 22).

Thus implantation of gene *A1* has led to a distinct therapeutic effect.

Discussion of the results. Along with anticipated results the experiments have brought about additional data that prove not only unexpected but also demanding further explanations. The main integral result consists in a fundamental possibility to influence the process underlying mass pathologies by means of implantation of outside molecular constructions with a corresponding gene. However this general conclusion somehow conceal the peculiarities specifying the process. Let us distinguish and analyse them.

First that stands out is a low correlation of the results obtained in the culture outside the organism and in the animals, i. e. on the organism level. It has been expected this way or the other. However it should be emphasized once again as applied to gene therapy of mass pathologies Estimation of the recombinant plasmids for the absence of random mutation (in a broad sense of the notion) defects in them which are possible under assemblage of complex construction is real in the systems *in vitro*. Another general estimation of the processes using cultures of cells such as tissue-specificity of regulatory elements, effects of subsequent transfections etc. is also possible. Cell cultures are far and away helpful in this regard. But further extrapolations with them on the organism are not effective.

The next group of phenomena may be characterised as an ambiguity of the expression of the recombinant molecules. It manifests in the following. On the one hand different molecular constructions containing one and the same gene under different promoter-enhancer systems even if they are not tissue-specific express differently in one and the same cells. On the other hand one and the same recombinant molecule (again if its gene is under nontissue-specific promoter-enhancer system) is differently expressed in cells of different tissues.

Most likely it testifies to a conditionality of the term «nontissue-specificity» itself. Such nonspecificity is only of a qualitative character — the expression in all types of cells. Quantitative characteristics of the expression are nevertheless tissue-specific — in some tissues the expression is higher, in some — lower.

Then there follows a group of phenomena that may be called biological heterogeneity of the expression.

As is stated above there is always a high degree of heterogeneity in cells by the expression of the implanted gene in different cells of one type (for example hepatocytes) in one organ (liver in this case) in one specimen (particular experimental animal under one population of identical recombinant molecules). Special studies have shown that at least for the liver such heterogeneity is not connected with a nonuniform distribution of the implanted material. In some dozen seconds it spreads uniformly over the organ. Moreover, the material from different liver sections has the same spread of expression as in the place of injection.

Finally, even in the cultures of cells where the population is maximally levealed off both by the fussion stage and spatially (monolayer) one observes the same heterogeneity of the expression in principle.

Therefore we have to admit the presence of the individual cell heterogeneity in the quantitative characteristics of the expression of the gene implanted from outside both in the culture and (which is more important) in the organism. But heterogeneity by this criterion takes place in the organism also — a quantitative characteristics of the expression of the gene implanted to different organisms (one species and one line) are also different. It is here very essential that the character of the cell heterogeneity, i. e. distribution curves (see section III) are different in different individuals. So, along with a cell heterogeneity we observe also an individual organism heterogeneity with regard to the expression of the gene implanted from outside.

The phenomenon in question seems to be exhausted by these two types of heterogeneities. But the reality proves much broader. Age variability in the level of expression is demonstrated above. In its pure form, i. e. as an expression of only one implanted gene it decreases with age (though individual types of spreading are present). Analysis of the changes in different metabolic chains induced by the implantation of one key gene has shown that there are processes which are more enhanced in the old organism than in the young one.

That is why it is so necessary to take into account the age heterogeneity by the same criterion — expression of the implanted gene and processes associated with it. Besides experimentally established types of heterogeneity one may predict (postulate) the fourth type of heterogeneity. It is specified by a physiological state of the organism. Due to this type of heterogeneity a response of one and the same organism in one and the same age will change depending on its state — stage in the development of the main disease, the presence of accompanying pathologies, stress, nervous breakdown etc.

Biological heterogeneity is, apparently, underlain by two phenomena. First, this is different competence of different cells. As a result recombinant molecules penetrate not in all cells but if penetrate then in different quantities. Second, in different cells even if the number of penetrated recombinant molecules is the same it is hardly possible to expect their similar expression. It is well known that any organism's own gene is somewhat different by the level of its expression. Different cells display different total level in synthesis of DNA, protein etc. The existing differences wholly depend on the state of the cells. But the state of the cells will be inevitably affected by the state of the organism, its individual peculiarities, age etc. State of the cells will affect their competence. All these factors specify biological heterogeneity of the expression under implantation of recombinant molecules *in vivo*.

All the above stated evidence for the necessity to individualize gene therapy of mass pathologies. Biological heterogeneity this way or the other should be taken into account under classical hereditary diseases. Biological heterogeneity in view of many metabolic chains that are being envolved into the process prove damaged becomes defining in many respects. So, it demands development of special methods for estimation of such heterogeneity and subsequent treatment with regard for the testing results. Each man prefers his own molecular construction optimal for him only though it may have one and the same key gene. Different implantation systems are necessary for different people. The quantity of recombinant molecules for different people are also different.

Individualization of gene therapy is a new problem not yet stated. The sooner its development will start the better.

A system of gene implantation should be treated with great care If the gene is implanted only once it will be modified by the above stated phenomena. However gene therapy of mass pathologies will require frequently (if not in most cases) repeated implantations [1, 64]. In this case one may expect surprises. As it was stated above even under transitory expression (determined by the vector construction) under repeated gene implantation one may expect not wave but additive effect. Though such situation is observed only in cell cultures, but two moments of the experiments suggest the idea that this is possible in the organism.

First, repeated implantation of gene has also revealed a biological heterogeneity not on the distribution curve but at a level of different subpopulations.

Second, this effect is observed only when the cultures are implanted under half-selective conditions, but these are peculiarities of their physical state. Possibly, in some individuals due to their specific physiological state this situation may arise in the organism. Further experiments will permit refining this supposition.

The idea of a «key» gene that should exist even in diseases envolving different physiological, biochemical and molecular processes and thus not several genes but numerous gene ensembles is a fundamental element of gene therapy of mass pathologies and it should be practically confirmed.

Strictly speaking, even under classically monogenic diseases a defect of only one gene inevitably causes disturbances of many physiological, biochemical and molecular processes and, thus functioning of gene ensembles that is generally accepted. It is enough to see what is going on in the organism under tellasemy, classical hereditary diabetes, hypercholesteremy specified by the defect of either structural or regulatory zone of gene *apoA1*, etc. In this case a key role of one gene in the disturbances involving the whole organism is evident. But in case we do not know that atherosclerosis is caused by the defect of gene *apoA1* its role seems to us dubious. Then it is obligatory to find a really defected gene and attribute everything to it. But it would be valid only if the processes in the organism are independent. But they are not only dependent but also ranged by a degree of defect compensation. Something may compensate only itself, something — the whole process, and something all its «hierarchy» of processes. Otherwise organism cannot exist-defect of any gene would lead to its death. So the main problem is reduced to finding of such key gene which would compensate its hierarchy of the processes. For IDDM it is insulin gene (even if it is not genetically damaged under IDDM); for atherosclerosis it is *A1* though not in all cases of this pathology. Development of gene therapy of mass pathologies will undoubtedly give rise to studies on finding «key» gene for this disease.

Analysis of the above described gene *apoA1* implantation has revealed an obvious quantitative discrepancy of the human protein *A1* synthesized in the animal's organism and the response of other metabolic chains on it. This discrepancy is most evident if to compare the quantity of the tested human *A1* protein and an increase of the quantity of lipoprote-

ids containing a total protein *AI* in experimental animals with regard to control. The content of protein *AI* in rabbits (on the example of Japaneese white rabbit) amounts to about 37 mg per 1 kg of the animal's weight [65], while synthesis of human *AI* after implantation of a corresponding gene amounts to unities of μg per 1 ml of blood, i. e. is 1 mg per 1 kg of the animals weight. It is less than 3 % of animal's own protein. Increase of lipoproteids containing protein *AI* is some dozed percents.

A simple extrapolation of a level of *AI* synthesis on the base of the implanted gene on all the processes caused by this synthesis cannot explain the situation. However medicine has accumulated much information about discrepancies of this type. In view of the evident effect induced by administration of ATP, cocarboxylase, phospholipids («liposta-bil», «essentiale» drugs, etc.) the quantity of the administered preparations is only thousandth fractions of the quantity always normally present in the patient's organism. And clearance of such preparations is only some minutes. Dose disperancy cannot be adequately explained by formation of some specially active biological derivatives from the administered preparations. Since the quantity of the same products of organism's own origin is hundreds (and even thousands times) more than the administered ones, so a corresponding quantity of derivatives forms in this case. Evidently there should exist some additional explanation. Here we may admit a new type of regulation. The regulation that accounts for almost all effective events in the organism is based on the principles of the feedback with an oposite sign. If the quantity of the products increases the regulation decreases its synthesis, the quantity falls, the synthesis is enhanced. For the most of proteins there is a base constitutive synthesis that is permanent and is not subject to regulation. It is likely to be valid for metabolic processes. But the organism is the most complex tangle of correlating processes and their disturbance is a powerful destructive factor for the organism [66]. Fluctuations both outside and inside the organism cannot but lead to temporal change of these or other processes. All this causes a quantitative disbalance — a discrepancy of mass and energy flows between metabolic chains. The first stage for compensation of such disbalance is well known: it is reverse enzyme capacities. A part of enzymes is in its nonactive state and eliminates a disbalance of mass and energy flows either at the account of conformation transitions (instant response to disbalance) or at the account of modifications: methylation-dimethylation, phosphorylation-dephosphorylation etc. (fast response to disbalance). If the constitutive synthesis was really constitutive (i. e. permanent as a constant) then any reverse capacities of the enzymes would fall into disbalance too. In this case there must be a system reserving them in necessary quantities, the system that cannot be regulated since the bulk of genes practice a constitutive synthesis. The constitutive synthesis is determined as the one that cannot react on the changes in the organism. So, there should be a regulation of the constitutive expression. This regulation must be very prompt and necessarily a complex one. It may be called a regulation by stabilization «from the achieved». Variations of the synthesis from any constitutive promoter are possible and really take place within certain limits. Control of the expression cannot be limited presently by only one promoter (even in genes of constitutive synthesis). There are always other sequences involved into the control of the expression. Balancing of metabolic chains being constantly changed under the action of internal and external disturbances may be realized by the regulation of their stabilization by the central (most essential, dangerous etc.) process. This will be a regulation «from the achieved». Let us make an example. As a result of any disturbance but within the regulation there changes the content of protein *AI* (even locally in the liver). This change causes either increase or decrease of the quantity of expressed (i. e. open for recognition) receptors (for *AI*, HDLP, cholesterin or some other that is not significant for the

regulation «from the achieved»). Such changes does not require new synthesis. It will be also regulated by the same mechanism «from the achieved». But it is a long process while ready molecules of the receptor are reserved in the membrane. Depending on *AI* (HDLP, cholesterin etc.) their quantity is simply redistributed. Closed (submerged) molecules transfer into an open state accessible for the reception or vice versa. Here is also a feedback but with a similar sign. The more is *AI* (HDLP, cholesterin etc.) the more are the receptors (the reserved ones have transfered from the submerged into the open state). The less is *AI* (HDLP, cholesterin etc.) the less is the quantity of open receptors (part of them is submerged into the membrane). But this process cannot be continuous since the quantity of receptors is limited so the range of the response «from the achieved» is also limited. Receptors as connected with their ligands sustain expression of the genes in many fundamentally connected metabolic chains. So the synthesis of proteins from the associated processes should be oriented on the main, essential, dangerous etc. metabolic process. Enhancing of the process enhances the rest. Its weakening induces the same reaction. The process itself should be kept at a level «from the achieved». It makes the system stable. Otherwise the system will go wrack because of frequent oscillations. A range of the positive response (i. e. the whole molecular machine — a regulatory region of genes, receptors and their exposure, a signal from the receptors to the regulatory zone, a ligand recognized by the receptor an so on) will be this very range of the norm within which each cell of the organism is able to resist adequately and correspondingly to variations of outer and inner character.

In this variant small amounts (with regard to the available in the organism) of either administered or locally synthesized product may exert an adequate reaction if they come to the reception system. The reception system allows for an increase of the product synthesis in response to the appearance of the same product with simultaneous enhancement of other metabolic processes connected with it. But this level will exist till the next compensation and then the system will become stable on a level «from the achieved» etc.

Further studies will show if the supposition is valid.

Regulation of the expression is a principle moment for the gene therapy of mass pathologies. It is solved by using constitutive promoter-enhancer system (frequently nontissue-specific). But it is not a regulated expression. If to clarify the types of regulation in its usual sense and in view of the gene therapy one may single out the following three principles: 1 — «as called for»; 2 — «as required»; 3 — «at will».

The first one means that the regulation is expecting its time. For example under embryogeny these or other groups of gene come into action alternatingly in the course of the development.

The second principle is a usual adaptive regulation. It means a response to some action.

The third type is the use of the potentialities hidden in the second principles for the tasks of gene therapy. Placing the required gene under the regulatory sequence induced by the hormone or heavy metal we may willingly actualize its expression by introducing the inductor. It is a widespread model in the experiments on cell cultures and laboratory animals. But in its present form it is not suitable for people since we cannot intoxicate organism with heavy metals or destroy it with hormones for the sake of treatment. In due course there will be created systems for actualization of genes «at will» applicable for human. Without going into particulars we may state that such works are in progress though on cell cultures so far. But for the time being there remains a constitutive synthesis with the regulation «from the achieved» (in case future works confirm it).

Classical hereditary diseases are on this case in more favourable conditions. There at least it is enough to implant gene with its own re-

gulatory region. The rest of the organism as genetically sound will accept it as a missing link of a single whole.

Finally it is very important and in future even strategic to demonstrate fundamental potentialities of gene therapy for age pathologies. The opened prospects to eliminate the grounds of the pathology in the ageing organism needs further analysis that is to be done. It is unusual in this case that gene implantation eliminates the damage that is a consequence of the ageing and thus is incurable in principle since the ageing is inevitable, irreversible, natural and entropy-conditioned process with the only one way — to the better world.

Against this background one tries to demonstrate a fundamental potentiality to parry the age pathology. Not the ageing on the whole but only one pathology. But even this chance makes the whole situation unsteady. If it is possible to restore only one function by gene therapy then why not two or ten? And in the long run a man has about as few as fifty thousand structural genes that is not so many. However not everything is so simple here. Only a detailed analysis and further experiments will unswer the questions stated by potentialities of gene therapy of mass pathologies.

These are brief results of the performed studies. As it is always with the new — the works rather state new problems then solve the old ones but gene therapy of mass pathologies is on the agenda now demanding not only attention.

Р е з ю м е. Описано комплекс досліджень, який дозволяє пройти шлях від ідеї до експериментального вирішення принципової можливості генної терапії масових патологій, на прикладі інсулінзалежного цукрового діабету та атеросклерозу.

Зроблено добір та аналіз регуляторних елементів, які дозволяють здійснювати експресію екзогенних генів незалежно від стану загальної клітинної регуляції. На клітинах різних тканин та організмів, а також *in vivo* показано експресію генетичного матеріалу, що вводиться (як модельного гена бактеріальної β-галактозидази, так і генів інсуліну та аполіпопротеїну високої щільності *AI*, які мають відношення до вищезгаданих патологій).

Відзначено неправомірність екстраполяції результатів, отриманих в культурі клітин, на тварин; показано неоднозначність експресії рекомбінантних молекул залежно від генного оточення і типу клітин-реципієнтів. Як в культурі клітин, так і в організмові виявлено індивідуально-клітинну гетерогенність кількісних характеристик експресії введеного ззовні гена. Крім того, відзначено індивідуально-організмову та вікову гетерогенність експресії екзогенної інформації.

Зроблено висновок про необхідність індивідуалізації генної терапії масових патологій.

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