
PROBLEMS AND PROSPECTS

Expression of Eukaryotic Recombinant Proteins and Deriving Them from the Milk of Transgenic Animals

Yu. M. Khodarovich^a, I. L. Goldman^b, E. R. Sadchikova^b, and P. G. Georgiev^b

^a*Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, 117997 Russia*

^b*Institute of Gene Biology, Russian Academy of Sciences, Moscow, 119334 Russia*

e-mail: khodarovich@mail.ru

Received June 29, 2012

Abstract—Expression in milk of transgenic farm animals is one of the most effective ways to produce eukaryotic recombinant proteins. In this review, we discuss the problems of the appropriateness of selecting this expression system for the production of proteins and the development characteristics of appropriate production processes.

Keywords: biotechnology, milk, recombinant proteins, transgenic animals

DOI: 10.1134/S0003683813090020

Preparations based on recombinant proteins, such as hormones, enzymes, blood factors, growth factors, and monoclonal antibodies (monaABs), are increasingly used in modern medicine. Global sales of biopharmaceuticals, which are mainly represented by recombinant proteins, have doubled in the period from 2004 to 2009 and amounted to 92 billion USD. By 2015, this figure is predicted to increase to 167 billion USD [1]. Growth in sales is mainly expected due to an increase in the share of drugs based on monaABs up to 79 billion USD in 2015. At the same time, the patent protection of many successful drugs is expiring, which leads to increased competition among manufacturers. In these circumstances, more and more attention is paid to the reduction in the primary cost of recombinant proteins and improvement of their quality by selecting new more efficient expression systems.

The technology of industrial production of recombinant proteins (RPs) is a complex multistep process that involves the creation of a producer, development of RP purification technologies, organization of monitoring systems for the parameters of the production process, and quality control of preparations. Today, the establishment (or extension) of production based on cultures of eukaryotic cells requires a lot of time (two to five years) and financial expenditures (50 to 500 million USD), depending on the production volume [2]. A very important step on the way of an RP-based therapeutic agent to the market is the approval

of the production technology by regulatory authorities, which require the technological process stability and the resulting product biosafety.

The development of a technological process that meets these requirements is greatly simplified if earlier approved technologies for RP production are used, because, in this case, the requirements for the production process and the approaches to solving technical problems are already known. When fundamentally new approaches for RP production are used, the time and financial expenditures associated with the official approval of the technological process increase manifold. This can largely explain the fact that new technological platforms for protein production, such as those based on the use of transgenic animals and plants, practically have not been used earlier despite their economic attractiveness. For example, all ten biopharmaceuticals approved in 2008 in the United States and Europe were obtained using *E. coli* or yeast cells or mammalian cell cultures (CHO) [3].

However, the situation has begun to change recently. For example, a vaccine obtained in a baculoviral expression system and a recombinant human antithrombin produced in the milk of transgenic goats was first approved for use in the United States in 2009. The production of the recombinant C1 inhibitor, isolated from the milk of transgenic rabbits, was approved for use in Europe in 2010. Thus, it is seen that the use of transgenic animals is a competitive modern approach to the industrial production of recombinant proteins.

This review provides a comparison of different RP expression systems, discusses the advantages and disadvantages of RP expression in the milk of transgenic

Abbreviations: GEC, genetic engineering construct; G-CSF, granulocyte colony-stimulating factor; monaAB, monoclonal antibodies; RP, recombinant protein; EDTA, ethylenediamine tetraacetic acid; CaP technology, calcium phosphate technology; CHO, Chinese hamster ovary cell line; FDA, Food and Drug Administration (United States); GAP, Good Agricultural Practice.

animals, and considers the characteristics of the creation of such production.

MODERN SYSTEMS FOR RECOMBINANT PROTEIN PRODUCTION

At present, there are many different approaches to obtain RPs; however, only some of them are used in industrial production. The main characteristics of industrial expression systems are summarized in Table 1. The first RPs were obtained using bacteria. The bacterial expression system is considered to be the least expensive for the production of ordinary RPs without posttranslational modifications. Today, approximately half of RPs are produced on the basis of bacterial cells [4].

Under the conditions of strong competition among producers, even a slight effect of posttranslational modifications on the protein function can improve its quality and therefore provide a competitive advantage for the manufacturer of the given preparation. For example, the absence of glycosylation of human interferon-beta produced with the use of bacteria leads to a tenfold decrease in the specific activity. This disadvantage is compensated by administering patients with higher doses of the protein produced by bacteria with a greater frequency. According to the available data [5], there are no significant differences in the outcomes of treatment with interferon- β of bacterial and eukaryotic origins. However, although the prokaryotic interferon- β appeared on the market earlier, its current sales are less than a quarter of the world market of interferons- β [6]. In general, there is a tendency toward gradual replacement of the production of recombinant therapeutic proteins by using bacteria with production based on the use of eukaryotic, yeasts, and mammalian cell cultures.

The yeast expression system allows for the obtaining of glycosylated proteins. However, in yeast cells, long oligomannose glycosylated chains are synthesized, which do not always ensure sufficient biological activity of RPs in the case of glycosylation. In addition, in the human body, proteins with oligomannose chains bind to the mannose receptor and undergo rapid clearance. Typically, although not always, the level of RP expression in a yeast system is lower than in a bacterial system. For example, in *Pichiapastoris*, yeast cells often allow for the obtaining of RPs at a concentration of 10 g/L of culture medium.

Recombinant proteins obtained in insect cells have the majority of posttranslational modifications characteristic of higher eukaryotes. However, in this expression system, posttranslational modifications, adversely affecting the biological activity and immunogenicity of the protein, may also take place. This is a relatively new technology, and companies, such as Pfizer, Wyeth-Ayerst (United States), Bayer (Germany), and Human Genome Sciences (United States), are working on improving it. In particular, the

problem of removing some of the approximately 100 genes constituting the baculoviral genome (which is used to infect insect cells for RP expression) is studied with the purpose of simplifying the product purification process. Currently, baculoviral infection is a rare component of the expression system, which is basically tried to be used for the production of vaccines, because, in this case, the immunogenicity of the protein determined by the posttranslational modifications is not an obstacle to the use of the drug.

An interesting system from the economic point of view is the expression system based on transgenic plants, which makes it possible to obtain a very inexpensive RP. The organization of production in this case is cost- and time-effective. The main obstacles in using this technology are the difficulties associated with the isolation of RPs from the plant mass, the fact that posttranslational modifications in plant and mammalian cells are different, and the unresolved problems of the biosafety of products. The last issue is the most important for the development of this technology. There are already several cases of the accidental introduction of transgenes into food products. For example, in 2000, the genetically modified Starlink corn (Aventis, France), which was not approved for human consumption, was found in foodstuff in the United States. It required about 1 billion USD to recall these products, clean storages, and pay all claims [7]. In 2002, the ProdiGene company (United States) paid a fine of 250 000 USD for getting genetically modified maize on fields with conventional crops and 1 million USD for the remediation of soil pollution. Currently, genetically modified plants are used in agriculture; however, there is no single drug protein derived from transgenic plants. Apparently, it will take time to solve the problems of biosafety, allergenicity, posttranslational modifications, and organization of RP production from transgenic plants. Apparently, the most promising medical use of this technology in the near future will be the production of various edible vaccines.

The expression of proteins in mammalian cell cultures makes it possible to produce complex RPs with posttranslational modifications, which are the closest to the modifications of human proteins. Such proteins usually exhibit biological activity and low immunogenicity and can therefore be used as a medicine for a long period of time (sometimes for a lifetime). For many proteins, it is practically the only expression system that can yield products of the desired quality. For example, all antibodies for therapeutic administration are produced using mammalian cell cultures.

Besides the above, there are other less common expression systems of recombinant proteins. Expression systems of recombinant proteins based on the use of new microorganisms are developed, and cell-free synthesis systems are improved. From the above examples, it is clear that any expression system has not only

Table 1. Comparison of various RP expression systems

Expression system	Advantages	Disadvantages	Product quality control parameters	The production cost of 1 g of protein, USD	Examples of use
1	2	3	4	5	6
Bacteria	Numerous cases of use for the production of RPs, a well-known genetics of the organism, easily and inexpensively grown cells, inexpensive growth media, very rapid achievement of high levels of expression (sometimes for 5 h), fast-growing cells (division time, 20–30 min)	Proteins are usually not secreted (thus, the cell destruction stage is required, which complicates purification); the preparation contains endotoxins and is microheterogeneous; posttranslational modifications are absent; there is the probability of protein misfolding; the protein can be damaged during isolation from cells	Removal of endotoxins, DNA, host cell proteins, and various protein variants containing terminal methionine. It is also necessary to remove the misfolded protein and the protein partially cleaved by proteases	No data	Insulin from Eli Lilly and Company (United States)—the first preparation obtained using recombinant DNA technology, approved by the Food and Drug Administration. Genentech, Inc., uses <i>E. coli</i> for the production of the human growth hormone
Yeast	Yeast is commonly regarded by regulatory authorities as “safe” organisms; a long history of use, well-understood genetics, high-level expression in a relatively short (2–8 weeks) period; cells grow rapidly, the growth medium is inexpensive, proteins are usually folded correctly and may have posttranslational modifications; and the protein is secreted which facilitates its extraction	Overglycosylation can completely disrupt the biological activity and reduce the safety, effect, and clearance; RPs may contain immunogens or antigens; RPs are not always folded properly	Protein glycosylation profile	50–100	For hundreds of years, <i>S. cerevisiae</i> and <i>S. pombe</i> were used in large-scale fermentation processes for the production of beer. Yeasts are used for the production of vaccines, such as hepatitis B vaccine, and for obtaining human insulin. Aventis (France) produces human serum albumin using yeast
Insect cells	Presence of posttranslational modifications; the protein is folded correctly; the protein is secreted; a relatively high expression level is reached sufficiently quickly (within 4 weeks); baculoviruses are safe for humans	Insect cells are used for expression for a short period of time, and there are not many examples of their approval by regulatory authorities; they grow slowly; the growth medium is expensive; baculoviral infection is an additional step in the technological process; it contains immunogenic proteins of the host cell; glycosylation is sometimes incorrect; mammals can be infected with viruses	Removal of baculoviruses and host-cell proteins, posttranslational modifications	No data	Novavax Co. (United States) uses insect cell cultures for obtaining virus-like proteins for their use as vaccines and drugs, diagnostics, and viral drug delivery systems. Vaccines from Novavax are in clinical trials. Glaxo-SmithKline (Great Britain) manufactures the Cervarix vaccine using insect cells. This is the first and to date the only biopharmaceutical produced in insect cell cultures

Table 1. (Contd.)

Expression system	Advantages	Disadvantages	Product quality control parameters	The production cost of 1 g of protein, USD	Examples of use
1	2	3	4	5	6
Transgenic plants	Easy storage of the seed bank, production scale, and a good level of expression (up to 1 kg of recombinant protein from one acre of field); the genetics of organisms was studied; none of the known phytoviruses infects humans; low production costs	New types of impurities may be present in RP preparations (soil fungi and bacteria, pesticides, herbicides, and plant metabolites); posttranslational modifications are different from cells produced by animals; plants contain potential allergens; biosafety issues are not resolved	Removal of viruses, injection of plants with pathogens	10–20	The ProdiGene company (United States) sold recombinant avidin and P-glucuronidase obtained with the use of transgenic maize cells as a reagent for diagnosis and research. In 2011 Vértia Bioscience (United States) announced the commercialization of nine human RPs that were obtained from transgenic plants. Of these, four proteins are intended for “human health” (lactoferrin, albumin, transferrin, and lysozyme). However, there are no references to the passage of clinical trials by these preparations. Presumably, these recombinant proteins will be used in cell culture media, in scientific research, and, probably, for the treatment of wounds
Culture of mammalian cells	As a rule, the folding of proteins and posttranslational modifications are correct; can secrete the protein; numerous examples of licensing the technology by regulatory authorities; the only way (except for technologies based on the use of transgenic animals) to express large complex proteins	Expensive medium, slow growth, may contain allergens and impurities from the serum used in the growth medium; difficult purification; expensive final product	Removal of viruses and DNA	300–3000	Currently, many different biopharmaceuticals, such as erythropoietin and interferon- β , and various antibodies are produced using mammalian cell cultures

attractive characteristics (simplicity and low cost of the protein, etc.) but also disadvantages. At the current level of molecular biology development, these defects can almost always be corrected. For example, numerous papers describing changing the nature of glycosylation have been published. Various methods for increasing the expression level of the target protein in different systems, etc., have been proposed [8–10]. Thus, the current scheme of selecting a platform for the expression of RPs can change dramatically in the near future.

CREATION OF TRANSGENIC ANIMALS PRODUCING RECOMBINANT PROTEINS

The idea of using the milk of transgenic animals for the production of RPs emerged in the mid-1980s almost simultaneously with obtaining the first transgenic animals. Since that time, this approach has been well studied. It has been shown that transgenic animals can thus produce virtually all proteins in a functionally active state and can therefore serve as an alternative to the use of mammalian cell cultures. A lactating mammary gland is a natural bioreactor that consumes the nutrients of the animal and uses them for the synthesis and secretion of the protein. This allows for a very high level of expression of RP (40 g per liter of milk) to be reached [11].

The first step in obtaining animals producing RPs in milk is the development and creation of an appropriate genetic engineering construct (GEC). The GEC structure determines the level of RP expression in milk. The main objective is to reach a high level of RP expression only in mammary gland cells, because the expression in other organs and tissues may affect the animal's health. For this reason, the regulatory regions of the genes that encode the major milk proteins (caseins and lactoglobulins) are usually used for the expression of RPs. Insulators are also often used in GECs, which are supposed to increase the level of transgene expression, provide independent expression regardless of the transgene integration site into the genome of an animal, and prevent corepression in case of multicopy insertions of the transgene into the genome [12, 13].

It should also be noted that there are constraints imposed by regulatory authorities on the presence of certain DNA sequences in a plasmid that is used for obtaining transgenic animals. For example, the AgResearch company (New Zealand) was allowed in 2002 to create transgenic animals that should not contain the following types of DNA: (1) viral sequences other than the replication origin site of SV40 or Epstein–Barr viruses, (2) bacterial sequences other than marker genes and their promoters, (3) genes of known receptors that interact with viruses, (4) genes conferring resistance to antibiotics of medical importance, and (5) genes associated with the transmitted form of encephalopathy (bovine spongiform encephalopa-

thy). In the future, these stringent requirements for the composition of GECs were relaxed. For example, in 2005 AgResearch received permission to use the puromycin resistance gene in GECs, and in 2007 the presence of recombinase recognition sites (LoxP-sites) and the polyadenylation signal of the SV40 virus in GECs was allowed [14].

Commercially available expression vector pBS1, which carries the regulatory region of the goat beta casein gene, including the tissue-specific enhancer and two first noncoding exons, are most often used for the production of transgenic mice, goats, and cows. Two copies of the insulator (1.2 kb) isolated from the chicken beta-globin locus are inserted upstream of the regulatory region of this vector. The construct also contains the 7-kb 3'-end region of the beta-casein gene. For the RP expression, cDNA of a gene is used, which is inserted at the *Xho*I restriction endonuclease site, localized in the second exon of the gene. In some cases, an entire RP-encoding gene with introns can be inserted if this gene is small. Usually, transgenic animals obtained using full-length genes show a more efficient expression of the RP than those obtained with the use of cDNA.

Currently, there are no standard vectors and approaches that provide a stable high level of RP expression in the mammary gland of transgenic animals. In view of this, an optimal strategy is to create a series of GECs differing in the regulatory elements that control the transcription and the presence (absence) of introns in the coding region, which show different efficiency when tested in transgenic mice. This approach makes it possible to identify any potential negative effect of RP expression on animals' viability and lactation. An optimal GES variant, selected with the use of transgenic mice, will with a high probability be the most effective in the production of large transgenic farm animals.

An RP concentration in milk greater than 1 mg/mL is generally considered commercially attractive. Numerous cases of RP production in the milk of large animals when the RP concentration exceeded 10 mg/mL were described. Table 2 shows examples of production of different RPs in the milk of transgenic goats. It can be seen that, in some cases, the level of RP in the milk was relatively low. Typically, this is due to either an inappropriate integration site of GEC or the absence of testing in transgenic mice. In some cases (e.g., G-CSF), a high level of RP expression can reduce the survival of animals. As a result, only transgenic animals with a low level of RP expression can survive and produce offspring. It should be borne in mind that, after each RP reaches a certain level of expression, lactation is disturbed. Recombinant proteins can "clog" the secretory apparatus of mammary gland cells, cause cell death, or affect the development of the mammary gland as an organ. In view of this, the main task is to reach an optimal, rather than a maxi-

Table 2. Efficiency of production of different recombinant proteins with milk of transgenic goats:

Gene	Promoter	Expression level, mg/mL	Reference
MonoAB	Goat β -casein	14	[16]
Lactoferrin	Same	10	[13]
Antithrombin	"	0.09–12.5	[17]
Butyrylcholine esterase	"	0.1–5.0	[18]
α -Phetoprotein	"	0.6–1.1	[19]
Lactoferrin	"	0.77	[20]
Lysozyme	Cows alpha-sl-casein	0.27	[21]
G- CSF	β -Casein of goats and cows	0.07	[22]
Growth hormone	Goat β -casein	0.07	[23]
G- CSF	Same	0.05	[24]
Factor IX	Cow β -casein	9.5×10^{-5}	[25]

num, level of RP expression, at which the animal is healthy and stably produces milk [15]. For example, to produce human albumin, transgenic cows with an albumin concentration in milk varying from 1 to 40 mg/mL were obtained [11]. In all cows with higher levels of albumin expression, the lactation period was reduced, whereas in animals with an RP production level of 1 mg/mL lactation did not differ from the norm.

The next task is to choose an optimal animal producer of RP and a method for the introduction of relevant DNA into its cells. Table 3 shows the time required for creating a herd of transgenic animals of different species and the average annual milk yield per one such animal. Although the greatest amount of milk in domestic animals can be obtained from cows, the time spent on the creation of a herd of transgenic cows producing RPs is too long. Usually, transgenic goats are obtained, which provide an optimal ratio between the consumed time and the milk yield. In the case of small production volumes, transgenic rabbits

can be used, which reduces the time required to create a herd.

The creation of large transgenic animals itself is still a technically difficult, time consuming, and risky procedure. Many different methods for producing transgenic animals were proposed, including the use of viruses, transformation of spermatogonia and spermatozooids, use of transposons and homologous recombination proteins, etc. [26]. All these methods have certain disadvantages. For example, when using lentiviruses, it is impossible to work with genes containing introns; the gene size cannot exceed 8000 bp. In addition, only one GEC copy is usually integrated into the genome, which may be insufficient to reach the desired level of expression. The incubation of DNA with spermatozooids gives poorly reproducible results, and the DNA sequence in the genetic construct inserted into the genome is often changed.

In practice, the *method of DNA microinjection into the pronucleus of a zygote* is still used for large farm animals. The effectiveness of this method significantly differs for different animal species: it gives good results in mice, rabbits, and pigs but is much less effective in large animals [28]. This phenomenon can be explained by the following: the smaller number of animals in a litter hampers collecting a sufficient amount of zygotes for injections; it is difficult to "catch" the proper stage of zygote development; and the pronuclei of large animals can often hardly be distinguished, and the centrifugation of zygotes may require their visualization. Moreover, the efficiency of transgene integration in large animals is less than, for example, in mice. As a result, to obtain two or three transgenic cows, at least one year of work with a herd of about a thousand heads may be required, which is particularly intensified during periods of estrus. Various methods existed for increasing the efficiency of the microinjection method for producing transgenic animals, but all of them were either ineffective or poorly reproducible [29.30] and are not used today.

In recent years, large transgenic farm animals have been obtained using the *reproductive cloning method*. It is based on the possibility of creating an animal from an enucleated oocyte, into which the nucleus of a somatic cell is transferred. The insertion of a transgene into the oocyte using standard techniques (transfec-

Table 3. Time required to obtain herds of different transgenic animals and annual volume of milk derived from them [27]

Index	Rabbit	Pig	Sheep	Goat	Cow
Gestation period, months	1	4	5	5	9
Age of puberty, months	5	6	8	8	15
Time elapsed between the gene transfer and the first lactation, months	7	16	18	18	33
Number of offspring, heads	8	10	1–2	1–2	1
Average annual milk yield per animal, L	15	300	500	800	8000

tion or electroporation) before the nucleus transfer yields a transgenic animal. The cells in which the transgene was inserted are selected using antibiotics: the expression vector carries a gene conferring resistance to an appropriate antibiotic as a selection marker. The negative consequence of this approach is the expression of the antibiotic resistance gene in the derived transgenic animals.

The most important parameter that determines the method efficiency is the selection of cell donors of nuclei for transfer. Firstly, the nuclei of different cells vary in their ability to undergo reprogramming in the oocyte cytoplasm. In the experiments on the nucleus transfer described in literature, the number of born animals relative to the number of transplanted embryos ranged from less than 1 to 83%, which was mostly determined by the difference in the type of cells used as donors of nuclei and the conditions of their cultivation. Secondly, the selected cells should have a high ability to form a culture from a single cell, because individual cells must be able to develop in colonies during the selection of the clones stably transformed by the transgene. The most commonly used cells are fetal fibroblasts, which can undergo 30–50 divisions.

The main problem of the reproductive cloning method for obtaining transgenic animals is a high death rate of zygotes and embryos developing from them. This is apparently due to an incomplete reprogramming of the genome of the nucleus transplanted into the oocyte cytoplasm; currently, there is no solution to this problem [31]. The effectiveness of obtaining transgenic animals by this method can be estimated by the typical results presented in [32]: 2961 reconstructed zygotes were transplanted into 18 pigs. Six females were pregnant and gave birth to 15 piglets, 11 of which carried the transgene. It should be noted that the animals born as a result of a nuclear transfer often have lethal developmental deviations which further reduces the efficiency of the method. However, transgenic sheep [33], cows [34], and pigs [32] have been obtained to date using this method.

The main advantages of the method of obtaining transgenic animals by nuclear transplantation are the possibility of preselection of cell donors of nuclei that effectively express RPs, as well as a high percentage of transgenic animals in the progeny, which makes it possible to significantly reduce the number of recipient animals and, accordingly, the cost of obtaining transgenic animals. As a result, despite the fact that the nuclear transplantation procedure is labor-consuming and requires specialized equipment and highly skilled personnel, it is more preferable than the microinjection method and is today the basic technique to obtain large transgenic animals in many companies.

In recent years, reports on the development of promising new methods for producing transgenic animals have been published. One of them is *stable trans-*

formation with the transgenic of stem cells that are precursors of oocytes. After the transplantation of these cells into the ovaries of adult mice, 30% of born transgenic mice contained the transgene [35]. A similar approach is the transformation of stem cells that are precursors of sperm cells and their subsequent transplantation into the seminiferous tubules of infertile males [36]. At present, the prospect of using these new methods is unclear.

Another interesting approach that has emerged in recent years is to *increase the efficiency of the transgene integration using highly active transposases*. The gene encoding the transposase and the transgene flanked by the terminal repeats of the transposon are coinjected into the zygote. As a result of the reaction catalyzed by transposase, a single copy of the transgene is inserted into one or more sites in the genomic DNA of an animal. It is believed that, to simplify the control of inheritance, it is necessary to obtain animals whose genome contains only one transgene integration site; this can be ensured by crossing transgenic and nontransgenic animals. This approach has already been used to produce transgenic pigs [37]. The efficiency of the transgene integration in this case depends on the transposon type, the transgene length, the injected DNA concentration, and the injection site. It is noteworthy that the use of high-activity transposases yields a high percentage of integration (approximately 50%) even in the case of coinjection of plasmids into the zygote cytoplasm, which greatly facilitates the obtaining of transgenic animals [37]. At present, it is unclear how this method can be used for animals producing RPs in milk, since there is no clear evidence which level of RP production in milk can be reached when a single copy of the transgene is used.

PRODUCTION CREATION CHARACTERISTICS

Currently, there are only two enterprises producing RPs from the milk of transgenic animals. These data can provide a general idea regarding the organization of such facilities.

Much attention is paid to the health of animals producing RPs. First of all, animals should not have diseases caused by prions [38]. Prions are a special class of infectious agents composed of proteins only and containing no nucleic acids, which cause severe diseases of the central nervous system of some farm animals. This disease can be transmitted from animals to humans; since the infectious agent has a protein nature, the complete purification of RPs from prions is a difficult problem. Since the incubation period in this disease ranges from one to four years, the most appropriate approach is to work with animals that are guaranteed to be healthy. Australia and New Zealand are countries in which there are no scrapie (a disease of goats and sheep caused by prions), and transgenic

goats producing RPs are obtained in these countries or from herds of animals brought from these countries.

After obtaining a herd of animals that produce RPs, there is a risk of failure of the entire project because of their infection. To prevent such cases, the work with animals is performed according to the standards of Good Agricultural Practices (GAP). Transgenic animals are fed a standardized feed which does not contain animal products, their health is regularly monitored, and the visits of such animals by people are also controlled. A complete vaccination of animals is also performed, and a double fence around such a farm is constructed to prevent the entry of wild animals [39].

The regulatory authorities in Europe and the United States require manufacturers to meet certain biosafety measures. All animals that were used to generate transgenic animals and/or target product production cannot be used as a source of meat, milk, or other food [39]. There is also a danger of a horizontal transfer of a transgene (e.g., to a blood-sucking insect or soil bacteria). This primarily applies to antibiotic resistance genes, the transfer of which may lead to the development of bacterial resistance. In this regard, for example, the AgResearch company must perform periodic analyses of bacteria from soil near the habitat of transgenic animals for the presence of transgenes [14].

The process for milk collection is also standardized. It is known that the glycosylation profile of a protein in milk may depend on the lactation stage and somewhat vary among different animals in a herd. Therefore, to increase the homogeneity of the final product, batches of milk obtained on different days of lactation and from different animals are pooled.

There are several industrial protocols for the purification of RPs from milk, developed by different companies. The applicability and effectiveness of different protocols strongly depends on the RP properties. For the effective purification of a new product, modification of the existing methods or even the development of new ones may be required. At first, as a rule, fat is removed from milk by low-speed centrifugation (5000–10000 g). The resulting aqueous phase, called plasma, comprises casein micelles, water, lactose, and soluble noncasein proteins. Casein micelles can be removed by high-speed centrifugation; however, it should be borne in mind that they are the main protein fraction of milk and may contain the RP. If a significant portion of the target protein is included in the casein micelles, the latter should be destroyed to increase the yield of the RP. For example, the Bio-Sante company (United States) proposed a procedure for the purification of the RP from casein that is based on the use of calcium phosphate particles (CaP technology). Casein micelles are destroyed by introducing EDTA, after which new micelles are formed by adding calcium phosphate particles under conditions when the RP is removed. Caseins are precipitated from the mixture by high-speed centrifugation. This process of

micelle removal ensures a target product yield of about 90%; it can be easily performed on a mass scale and is inexpensive.

Another scheme was developed by PPL Therapeutics Ltd. (Great Britain) for the purification of the α 1-proteinase inhibitor from milk [40]. In this protocol, the stage of destruction of casein micelles is absent. Caseins are precipitated with polyethylene glycol and pelleted from the serum by centrifugation. Then, the remaining protein is concentrated by precipitation with polyethylene glycol. The final purification is performed by column chromatography in five stages. As a result, an almost pure target protein with a total yield of 44% is obtained. It is noteworthy that most substantial losses (30%) occur at the stage of removal of casein micelles. This method was also used for the purification of fibrinogen from milk [41]. However, in the last case, the RP coprecipitated with casein micelles and was rapidly damaged, apparently because proteolytic enzymes also coprecipitated with caseins. This problem was solved by breaking the association between casein micelles and proteolytic enzymes by the addition of lysine or another basic amino acid at a concentration of 10–200 mM.

The third method of RP purification from milk was developed by GTS Biotherapeutics (United States) for antithrombin III and includes flow filtration [42]. Unlike the previous case, it is not necessary to remove fat and casein micelles, which simplifies the process and increases the yield of protein. Flow filtration of milk removes caseins, fat, viruses, bacteria, and somatic cells. The purification process can be made continuous by constantly adding starting material and collecting the filtered liquid. The filtered liquid is then subjected to a series of chromatographic procedures. With this purification scheme, the yield of antithrombin III was 75–90%.

Note that the remaining steps of the production process, such as the control of purification of the preparation and the quality of the final product, do not differ substantially from similar procedures used in the production of RPs from eukaryotic cell cultures. Thus, owing to the already existing facilities, it can be assumed that many problems associated with obtaining RPs from the milk of transgenic animals have been solved.

ADVANTAGES OF THE PRODUCTION OF RECOMBINANT PROTEINS FROM THE MILK OF TRANSGENIC ANIMALS

Recombinant proteins, the properties of which are very similar to the properties of human proteins, can be derived from the milk of transgenic animals. Currently, these proteins are almost always produced using mammalian cell cultures. Table 4 shows a comparison of the key parameters of the production of recombi-

Table 4. Comparison of the production characteristics of recombinant antithrombin antibodies in the amount of 100 kg/yr using a mammalian cell culture and the milk of transgenic goats

Index	Mammalian cell culture	Milk of transgenic animals
Time required to create production, years	3–5	5–7 L
Reactor volume; number of animals	8500 L	35 goats
Costs of establishing production, million USD	50–300 (strongly vary in different sources)	10–50
Cost of 1 g of protein, USD	300–3000	105

nant proteins using transgenic animals and mammalian cell cultures [43, modified].

The data summarized in Table 4 show that the use of transgenic animals makes it possible to derive RPs at a lower cost and with a less cost of establishing production. In some cases, the cost of driving RPs is not significant. For example, the drug Rebif (human beta interferon) is sold in pharmacies of Russia at a price of 14000 rubles for 132 µg. Note that the cost of 132 µg of the protein (based on 2000 USD per gram) is eight rubles, and the total sales of this drug are approximately 2 billion USD/yr, which far exceeds the costs of establishing its production. When the price of a drug is much higher than its cost, it is expedient to use a standard mammalian cell culture for its expression, which will make it possible to rapidly and reliably establish production.

A somewhat different situation arises in the case of drugs with a high (milligram) dose. For example, the drug Herceptin® (monoclonal “humanized” (deliberately close in structure to human) antibody Her2) is priced at 70000 rubles for 440 mg, while the production cost of 440 mg of protein can be estimated (calculated as 2000 USD/g) to be 27000 rubles, which constitutes a significant proportion of the cost of the drug. In this case, the use of transgenic animals for protein production may be attractive. According to experts of the GTC Biotherapeutics company, the use of transgenic animals for the production of the drug Herceptin® will make it possible to reduce its final value by 30% [15]. The majority of proteins, the products of which in the milk of transgenic animals are currently established by biotechnological companies, are generics of the most widely sold drugs with a high contribution to the cost of RP production to the final cost of the therapeutic.

The relatively low cost of production organization in some cases may be a decisive argument in favor of deriving RPs using transgenic animals. This index becomes decisive at a low volume of sales of a drug (less than about 50 million USD a year) which happens where it takes several years of sales to recover the cost of establishing the corresponding production using cell cultures. Typically, this refers to drugs used to treat rare diseases. In this case, a drug can get the

status of an “orphan drug.” The US law provides for the granting of tax benefits and the exclusive right to sell the developed drug for several years to pharmaceutical companies that produce such drugs. Similar projects also usually expect to receive approval from regulatory authorities in a simplified manner, which is important when using a nonconventional technological platform.

It is possible to use transgenic rabbits to produce small amounts of RP in milk, which reduces the time and costs for its creation in comparison to transgenic goats. This approach was used to derive the drug Ruconest™/Rhucin® by the Pharming NV company (the Netherlands), which is used to treat a rare disease—hereditary angioedema.

Another factor that may affect the choice of transgenic animals to express proteins is the need for an RP with slightly different immunological properties than those characteristic of the existing drugs. This can be important for drugs used for a long time (lifetime), such as insulin. An immune response may develop in patients with time even to very highly purified drugs. In this case, the transition to a drug produced by another manufacturer and preferably obtained in another expression system will be required.

RISKS ASSOCIATED WITH THE ESTABLISHMENT OF PRODUCTION OF RECOMBINANT PROTEINS BASED ON TRANSGENIC ANIMALS

The organization of production of RPs from the milk of transgenic animals may fail for several reasons.

The creation of a large transgenic animal producing an RP is an expensive and unreliable process. In the case of both the microinjection method and the reproductive cloning technique, the low percentage of live transgenic animals relative to the number of transplanted embryos may lead to a project failure, especially when there is a time limit for carrying out such work. An animal may die without giving offspring, or it can secrete a low amount of protein with milk because of a poor integration site of the transgene. For example, the state-owned AgResearch company has stopped the work on the creation of transgenic animals

Table 5. Major biotechnological companies developing RP production from the milk of transgenic animals

Company (country)	Selling preparations	Formulations currently tested in clinical trials	Drugs in development
Pharming NV (Netherlands) www.pharming.com	Ruconest TM — Human C1 inhibitor	Human lactoferrin	Fibrinogen and type 1 collagen
GTC Biotherapeutics Inc www.gtc-bio.com (France)	ATryn—human antithrombin	No data	Blood coagulation factors: VIIa, IX, and VIII; albumin, α -1-antitrypsin; monoABs: anti-CD 137 and anti-CD20
AgResearch www.agresearch.co.nz (New Zealand)	No data	No data	MonoABs: herceptin and erbitux
Bio Sidus www.biosidus.com.ar (Argentina)	No data	Human growth hormone	Bovine growth hormone; human insulin, monoABs retuksimab, and etanercept

using therapeutic cloning because of a high mortality rate among the born animals [44].

The long process of production creation leads to uncertainties associated with the emergence of new more advanced systems for RP expression. For example, recent “humanization” of the yeast glycosylation machinery made it possible to obtain anti-Her2 monoAB in this expression system, which in the glycosylation profile and pharmacokinetics and biological activity is not inferior to a similar antibody obtained in mammalian cell cultures [45]. This development, in the case of successful completion of clinical trials, may reduce the appeal of projects on the production of similar monoABs in the milk of transgenic animals, launched several years ago by a number of companies.

Finally, a better drug may appear on the market during the time required to create a production based on the use of transgenic animals. For example, it has been found recently that the cellular cytotoxicity of antibodies with a glycochain increased approximately 100 times after the removal of fucose [46, 47]. The therapeutic properties of defucosylated antibodies are currently being studied, and, quite probably, some monoABs will be displaced in a while by defucosylated analogues. Thus, because of the long process of production creation, preparations from the milk of transgenic animals may not have time to pay off before the appearance of more advanced analogues.

Other risks of using transgenic animals for obtaining RBs include the uncertainty associated with the requirements of regulatory authorities for production. Although after the approval of the Ruconest and ATgup drugs (human antithrombin) relevant production was organized, there is a high probability of failure or a delay in the consideration of an application for the production of the drug using transgenic animals because of the small number of precedents.

MAJOR BIOTECHNOLOGICAL COMPANIES ENGAGED IN THE CREATION OF TRANSGENIC ANIMALS FOR OBTAINING HUMAN RECOMBINANT PROTEINS

Relatively few biotechnology companies are engaged in the development of production of RBs from the milk of transgenic animals. The major companies and their products under development are listed in Table 5. Apparently, the company that works most actively in this field is GTC Biotherapeutics Inc. (formerly Genzyme Transgenics Corporation, in 2010 was purchased by LFB Biotechnologies, France). This company has launched the first drug derived from the milk of transgenic goats—recombinant human antithrombin (ATryn)—to the market. This drug is used to prevent blood clots in patients with hereditary antithrombin deficiency. Today, the GTC Biotherapeutics company is developing the production of a large set of RBs from milk; many of these works are carried out by orders of third-party organizations.

Pharming NV is the second company that was able to bring a drug derived from the milk of transgenic animals to the market. The drug Ruconest, produced by this company, is the recombinant human C1 inhibitor derived from the milk of rabbits. This drug, as mentioned above, is used to treat a rare disease—hereditary angioedema. Currently, the company is developing the production of a relatively small number of new drugs derived from the milk of transgenic animals. The main efforts of this company are focused on expanding the indications for the use of the drug Ruconest. The Argentinian biotechnological company Bio Sidus obtained transgenic cows producing the human growth hormone (somatotropin) from milk to a concentration of 5 g/L. Currently, the comparative effectiveness of this drug is tested in a group of children with growth delays. Another company engaged in the development of RB production in milk of transgenic animals is New Zealand’s state-owned company AgResearch. It has signed an agreement with GTC

Biotherapeutics and Pharming NV on deriving analogues of the drug Herceptin (humanized monoclonal antibody to the Her2 receptor) from cow milk and Erbitux (humanized monoclonal antibody to the human epidermal growth factor) from goat milk. Other activities of the AgResearch company are aimed at improving the composition of milk for human consumption and the development of a technology for the directional insertion of genetic engineering constructs into the genome of transgenic animals by using site-specific endonucleases.

In addition to the successful companies operating at present, there are also examples of companies that have stopped the use of transgenic animals. The most famous example is the PPL Therapeutics company, which is known by the fact that it has funded the work on the cloning of Dolly the sheep. PPL Therapeutics has created a flock of 4000 sheep producing human alpha 1-antitrypsin. However, in 2003, a partner of this company—the Bayer firm—stopped funding this project in connection with the discovered side effects of the drug [48]. PPL Therapeutic was unable to fund the continuation of the project independently and ceased to exist. Another example is the development of the drug Protexia® by the PharmAthene company (Greece); the drug is a recombinant butyrylcholinesterase derived from the milk of transgenic goats, the expression levels of which in milk reach 5 g/L [49]. The main problem faced by this company is the impact of butyrylcholinesterase on the lactation of goats. In 2009, phase I of the clinical trials of the drug was completed. However, no reports of further trials have been published, which apparently means the cessation of work in this direction.

CONCLUSIONS

The expression of proteins in the milk of transgenic animals is a fairly well-studied technique that allows for the production of large amounts of RPs similar in properties to human proteins. The main competitive advantage of this technology is that the production cost is sufficiently low, and the most significant drawbacks are the unsolved problems of the organization of some stages of production and the long time required for its creation. Until recently, biotechnological companies have almost never used this technology for the production of RPs. However, currently, this situation is changing. The creation of production facilities obtaining RPs from the milk of transgenic animals has removed many issues associated with their organization. On the other hand, the expiration of patents for many therapeutics that are based on RPs has increased the competition among manufacturers. In this situation, the production cost of drug has become a much more important parameter than before. Today, more effective methods for obtaining large transgenic animals are being developed, which will reduce the time required for the establishment of production. Thus, it

is possible that the technology of deriving RPs from the milk of transgenic animals will be widely used on an industrial scale in the near future.

ACKNOWLEDGMENTS

This study was supported by the Ministry of Education and Science of the Russian Federation (state contracts nos. 16.512.12.2007 and 14.518.11.7040) and the Russian Foundation for Basic Research (project no. 10-04-01795-a).

REFERENCES

1. www.imarcgroup.com/global-biopharmaceutical-market-report-2010-2-15/
2. Farid, S.S., *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, 2007, vol. 848, no. 1, pp. 8–18.
3. www.biopharminternational.com/biopharm/Business/Biopharmaceuticals-Approval-Trends-in-2008/Article-Standard/Article-detail/632930
4. www.biopharma.com
5. Mazdeh, M., Afzali, S., Jaafari, M.R., *Acta Med. Iran.*, 2010, vol. 48, no. 2, pp. 83–88.
6. http://pharma.about.com/od/Sales_and_Marketing/tp/Top-Multiple-Sclerosis-Drugs.htm
7. Smyth, S., Khachatourians, G.G., and Phillips, P.W., *Nat. Biotechnol.*, 2002, vol. 20, no. 6, pp. 537–541.
8. Harrison, R.L. and Jarvis, D.L., *Adv. Virus Res.*, 2006, vol. 68, pp. 159–191.
9. Terra, V.S., Mills, D.C., Yates, L.E., Abouelhadid, S., Cuccui, J., and Wren, B.W., *J. Med. Microbiol.*, 2012, vol. 61, no. 7, pp. 919–926.
10. Castilho, A., Neumann, L., Daskalova, S., Mason, H.S., Steinkellner, H., Altmann, F., and Strasser, R., *J. Biol. Chem.*, 2012, vol. 287, no. 43, pp. 36518–36526.
11. Echelard, Y., Williams, J.L., Destrempe, M.M., Koster, J.A., Overton, S.A., Pollock, D.P., Rapijko, K.T., Behboodi, E., Masiello, N.C., Gavin, W.G., Pommer, J., van Patten S.M., Faber, D.C., Cibelli, J.B., and Meade, H.M., *Transgenic Res.*, 2009, vol. 18, no. 3, pp. 361–376.
12. Giraldo, P., Rival-Gervier, S., Houdebine, L.M., and Montoliu, L., *Transgenic Res.*, 2003, vol. 12, pp. 751–755.
13. Goldman, I.L., Georgieva, S.G., Gurskiy, Y.G., Krasnov, C.A., Deykin, C.A., Popov, A.N., Ermolkevich, T.G., Budzevich, A.I., Chernousov, A.D., and Sadchikova, C.I., *Biochem. Cell Biol.*, 2012, vol. 90, no. 3, pp. 513–519.
14. www.epa.govt.nz/Documents/GMD02028-decision.pdf
15. www.cosmosmagazine.com/node/4008/full
16. Pollock, D.P., Kutzko, J.P., Birck-Wilson, E., Williams, J.L., Echelard, Y., and Meade, H.M., *J. Immunol. Methods*, 1999, vol. 231, pp. 147–157.
17. Zhou, Q., Kyazike, J., Echelard, Y., Meade, H.M., Higgins, E., Cole, E.S., and Edmunds, T., *J. Biotechnol.*, 2005, vol. 117, pp. 57–72.
18. Huang, Y.J., Huang, Y., Baldassarre, H., Wang, B., Lazaris, A., Leduc, M., Bilodeau, A.S., Bellemare, A.,

- Côté, M., Herskovits, P., Touati, M., Turcotte, C., Váleanu, L., Lemée, N., Wilgus, H., Bégin, I., Bhatia, B., Rao, K., Neveu, N., Brochu, E., Pierson, J., Hockley, D.K., Cerasoli, D.M., Lenz, D.E., Karatzas, C.N., and Langermann, S., *Proc. Natl. Acad. Sci. USA*, 2007, vol. 104, pp. 13603–13608.
19. Parker, M.H., Birck-Wilson, E., Allard, G., Masiello, N., Day, M., Murphy, K.P., Paragas, V., Silver, S., and Moody, M.D., *Protein Expr. Purif.*, 2004, vol. 38, pp. 177–183.
20. Zhang, J., Li, L., Cai, Y., Xu, X., Chen, J., Wu, Y., Yu, H., Yu, G., Liu, S., Zhang, A., Chen, J., and Cheng, G., *Protein Expr. Purif.*, 2008, vol. 57, pp. 127–135.
21. Maga, E.A., Cullor, J.S., Smith, W., Anderson, G.B., and Murray, J.D., *Foodborne Pathog. Dis.*, 2006, vol. 3, pp. 384–392.
22. Freitas, V.J., Serova, I.A., Andreeva, L.E., Dvoryan-chikov, G.A., Lopes, E.S., E.S., Jr., Teixeira, D.I., Dias, L.P., Avelar, S.R., Moura, R.R., Melo, L.M., Pereira, A.F., Cajazeiras, J.B., Andrade, M.L., Almeida, K.C., Sousa, F.C., Carvalho, A.C., and Serov, O.L., *An Acad. Bras. Ciens.*, 2007, vol. 79, no. 4, pp. 585–592.
23. Lee, C.S., Lee, D.S., Fang, N.Z., Oh, K.B., Shin, S.T., K. and Lee, K., *Reprod. Dev. Biol.*, 2006, vol. 30, pp. 293–299.
24. Ko, J.H., Lee, C.S., Kim, K.H., Pang, M.G., Koo, J.S., Fang, N., Koo, D.B., Oh, K.B., Youn, W.S., Zheng, G.D., Park, J.S., Kim, S.J., Han, Y.M., Choi, I.Y., Lim, J., Shin, S.T., Jin, S.W., Lee, K.K., and Yoo, O.J., *Transgenic Res.*, 2000, vol. 9, pp. 215–222.
25. Huang, S., Zhang, K., Huang, Y., Chen, M., Li, H., Lu, D., Lu, J., Chen, Y., Qiu, X., Xue, J., and Zeng, Y., *Chin. Sci. Bull.*, 1998, vol. 43, pp. 1317–1319.
26. Gama Sosa, M.A., de Gasperi, R., and Elder, G.A., *Brain. Struct. Funct.*, 2010, vol. 214, nos. 2–3, pp. 91–109.
27. Houdebine, L.M., *Comp. Immunol. Microbiol. Infect. Dis.*, 2009, vol. 32, no. 2, pp. 107–121.
28. Wolf, E., Schernthaner, W., Zakhartchenko, V., Prella, K., Stojkovic, M., and Brem, G., *Exp. Physiol.*, 2000, vol. 85, no. 6, pp. 615–625.
29. Maga, E.A., Sargent, R.G., Zeng, H., Pati, S., Zarling, D.A., Oppenheim, S.M., Collette, N.M., Moyer, A.L., Conrad-Brink, J.S., Rowe, J.D., Bondurant, R.H., Anderson, G.B., and Murray, J.D., *Transgenic Res.*, 2003, vol. 2, no. 4, pp. 485–496.
30. Hirabayashi, M., Kato, M., Kaneko, R., Hirabayashi, T., Morita, M., and Hochi, S., *Exp. Anim.*, 2006, vol. 55, no. 2, pp. 131–135.
31. Zhao, J., Whyte, J., and Prather, R.S., *Cell Tissue Res.*, 2010, vol. 341, no. 1, pp. 13–21.
32. Cho, S.K., Hwang, K.C., Choi, Y.J., Bui, H.T., Nguyen, V.T., Park, C., Kim, J.H., and Kim, J.H., *J. Reprod. Dev.*, 2009, vol. 55, no. 2, pp. 128–136.
33. Schhieke, A.E., Kind, A.J., Ritchie, W.A., Mycock, K., Scott, A.R., Ritchie, M., Wilmut, I., Colman, A., and Campbell, K.H., *Science*, 1997, vol. 278, pp. 2130–2133.
34. Cibelli, J.B., Stice, S.L., Golueke, P.J., Kane, J.J., Jerry, J., Blackwell, C., Ponce de Leon, F.A., and Robi, J.M., *Science*, 1998, vol. 280, pp. 1256–1258.
35. Zhang, Y., Yang, Z., Yang, Y., Wang, S., Shi, L., Xie, W., Sun, K., Zou, K., Wang, L., Xiong, J., Xiang, J., and Wu, J., *J. Mol. Cell Biol.*, 2011, vol. 3, pp. 132–141.
36. Honaramooz, A. and Yang, Y., *Vet. Med. Int.*, 2010, pii: 657860.
37. Garrels, W., Mátés, L., Holler, S., Dalda, A., Taylor, U., Petersen, B., Niemann, H., Izsvák, Z., Ivics, Z., and Kues, W.A., *PLoS One*, 2011, vol. 6, no. 8, p. 23573.
38. Schmidt, S., *Nat. Biotechnol.*, 2006, vol. 24, no. 8, p. 877.
39. www.nytimes.com/2009/02/07/business/07goatdrug.html
40. Antonsen, K.P. and Lee, V.W., US Patent No. 6194553, C07K 14/81, 2001.
41. McCreath, G. and Udell, M., EI Patent No. 1115745, C07K 14/75, 2001.
42. Kutzko, J.P., Hayes, M.L., and Sherman, L.T., US Patent No. 6268487, A23J 1/00, p. 2001.
43. www.imptonline.com/articles/public/IPTFOUR92NP.pdf
44. www.i-sis.org.uk/death_rates_end_cloning.php?printing=yes
45. Zhang, N., Liu, L., Dumitru, C.D., Cummings, N.R., Cukan, M., Jiang, Y., Li, Y., Li, F., Mithell, T., Mallem, M.R., Ou, Y., Patel, R.N., Vo, K., Wang, H., Burnina, I., Choi, B.K., Huber, H.E., Stadheim, T.A., and Zha, D., *MAbs*, 2011, vol. 3, no. 3, pp. 289–298.
46. Shields, R.L., Lai, J., Keck, R., O'Connell, L.Y., Hong, K., Meng, Y.G., Weikert, S.H., and Presta, L.G., *J. Biol. Chem.*, 2002, vol. 277, no. 30, pp. 26733–26740.
47. Yamane-Ohnuki, N. and Satoh, M., *MAbs*, 2009, vol. 1, no. 3, pp. 230–236.
48. Baldassarre, H., Hockley, D.K., Lore, M., Brochu, E., Hakier, B., Zhao, X., and Bordinon, V., *Transgenic Res.*, 2008, vol. 17, no. 1, pp. 73–84.

Translated by M. Batrukova