Phenotype Manipulation by Gene Transfer in Animals - Review -

Y. Fujiwara, H. -M. Park and T. Muramatsu*
Department of Applied Genetics and Physiology, Graduate School of Bio-agricultural Sciences
Nagoya University, Nagoya 464-8601, Japan

ABSTRACT : Recent progress in molecular biology has made it possible to transfer genes of interest into cells and target tissues of living animals. This enables one to manipulate phenotype of cells and whole animals in selected and intended ways. The consequence of such gene transfer attempts have been the production of various types of "transgenic" animals that cannot be classified by classical nomenclature of exclusively either "transgenic" or "nontransgenic". Emphasis was placed on characterizing two transgenic categories, i.e., "transfectgenic and somatotransgenic" and "genuine transgenic" animals basically from a view point of their use for therapeutic purposes. Current state of art and possible solutions for problems encountered at present are discussed. (*Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 2 : 244-257*)

Key Words : Genuine Transgenic, Transfectgenic, Somatotransgenic, Localized *In Vivo* Gene Transfer, Xenotransplantation, Recombinant Protein Production

INTRODUCTION

The possibility that phenotype of cells and whole animals could be manipulated in selected and intended ways by introducing foreign DNA has long enchanted scientists. Recent progress in molecular biology has made it possible to transfer any genes of interest into cells cultured *in vitro* and even in target tissues of living animals *in vivo*, albeit at a low rate. Table 1 summarizes currently available gene transfer methodologies both *in vitro* and *in vivo*. For details, readers are referred to recent reviews (Murumatsu et al., 1998a, b). The obvious consequence of gene transfer attempts with those techniques is the production of a large number of cells and animals that belonged to somewhere between "transgenic" and "nontransgenic" cells or animals. Because many exceptions have thus emerged, the classical scientific terms and definitions to characterize them are outdated and no longer sufficient.

In the classical nomenclature, what has been proposed for defining such animals is exclusively "transgenic" or "nontransgenic". However, this is oversimplified, and reappraisal of transgenic nomenclature is definitely needed. Depending upon the target cells *in vitro* and *in vivo*, and the status of transgenes, Muramatsu et al. (1998b) have proposed new nomenclature of transgenic cells and animals as shown in figure 1. The classical term of transgenic animals herein is designated as "genuine transgenic animals" in which foreign genes are experimentally introduced and integrated in the genome of germ cells. As a result, the exogenous genes can be transmitted to progeny. A group of animals have been designated as "transfectgenic" in which transgenes are present in an episomal form. Therefore, transgenes of these animals disappear as the time goes by. Episomally present transgenes are the most likely consequence of the most *in vivo* gene transfer attempts. Because a number of *in vivo* gene transfer techniques have been recently developed, the production of tansfectgenic animals becomes easier and quicker than that of other types of transgenic animals. When foreign genes are transferred and integrated into chromosomes of somatic cells and

Table 1. Methods of gene transfer in cultured animal cells (*in vitro*) and tissues of living animals (*in vivo*)

In vitro	*In vivo*
Biological means	
Viral vectors	Viral vectors
Receptor mediation	Receptor mediation
Protoplast fusion	
Chemical means	
Lipofection	Lipofection
Calcium phosphate precipitation	
DEAE dextran mediation	
Polybrene mediation	
Physical means	
Microinjection	
Gene gun	Gene gun
Electroporation	Electroporation
Laserporation	Laserporation
Pricking	Direct injection
High frequency	High frequency

* Address reprint request to T. Muramatsu. Tel: +81-52-789-4076, Fax: +81-52-789-4077, E-mail: tatsuomu@agr.nagoya-u.ac.jp.

germ cells, they are referred to as "somatotransgenic" and "germinal tansgenic" animals, respectively. By analogy, the *in vitro* cultured cells are also presented under the corresponding transgenic names. Figure 1 also emphasizes how some types of transgenic cells and animals could be converted to others. The possibilities of such unidirectional conversion between transgenic cells and animals become realized through the production of sheep by nuclear transfer with a totipotent cell line (Campbell et al., 1996), and more popularly the "Dolly" sheep with a somatic cell (Wilmut et al., 1997). Via the "Dolly" pathway, the production of transgenic sheep, calves and goats has been reported (Schniek et al., 1997; Cibelli et al., 1998; Baguisi et al., 1999).

It is beyond the scope of this review to cover all the aspects of transgenic cells and animals and their use for a variety of purposes. Instead, basically from a view point of possible therapeutic applications, emphasis is placed upon two categories, i.e., "transfectgenic and somatotransgenic" animals, and "genuine transgenic" animals. Since only recently the production of "transfectgenic and somatotransgenic" animals becomes feasible, few examples are available in the literature. Therefore, in the following first half of this review, the way of production of "transfectgenic and somatotransgenic" animals, and regulation of foreign gene expression has been described, while in the second half, genuine transgenic animals have been documented in terms of the regulation of enhanced and tissue-specific transgene expression and their use for therapeutic purposes.

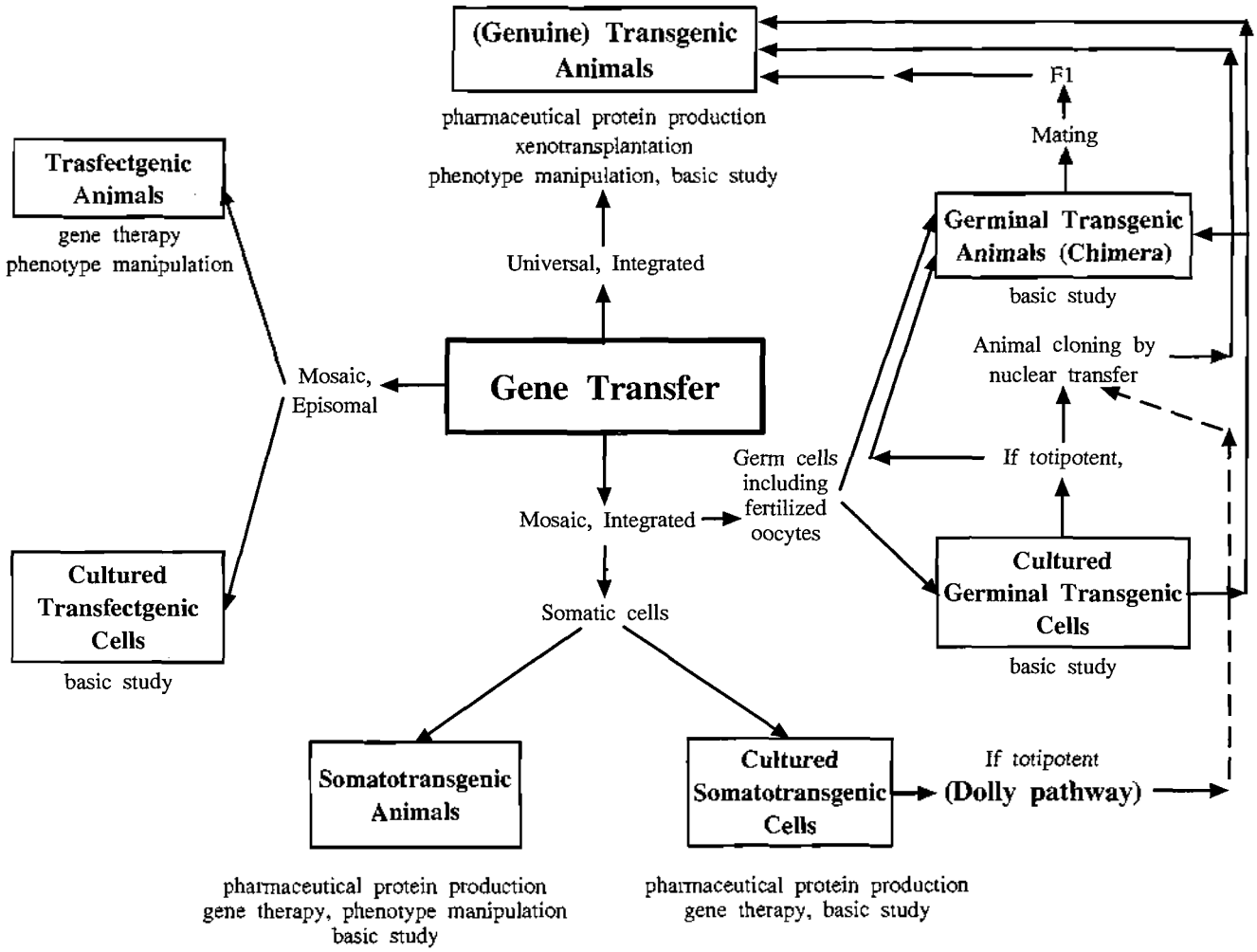

Figure 1. Classification of various transgenic cells and animals including humans to which foreign genes are transferred, and possible conversions from some types of animals and cells to others in conjunction with possible applications of these cells and animals for experimental and therapeutic purposes (Taken from Muramatsu et al., 1998b). The classical term of transgenic animals herein is designated as "(genuine) transgenic animals" in which foreign genes are experimentally introduced and integrated in the genome of germ cells so that the genes can be transmitted to progeny. Other types of cells and animals irrespective of the status of transgenes, are also classified under different transgenic names.

TRANSFECTGENIC AND SOMATOTRANSGENIC ANIMALS AND THEIR APPLICATIONS

Localized *in vivo* gene transfer techniques

In order to produce transfectgenic and somatotransgenic animals, foreign genes should be transferred somehow in tissues *in vivo*. Such gene transfer is usually limited to a localized area as a target. The most promising localized *in vivo* gene transfer (LIVGET) technique is the use of biological means, i.e., viral vectors including retroviral, adenoviral, and adenoassociated viral vectors. Mulligan (1993), Mitani and Caskey (1993), and Chong and Vile (1996) have pointed out that the use of the retroviral vectors has often been restricted because of (1) the dependence of retrovirus entry into cells on the existence of the appropriate viral receptor in target cells, (2) necessity of cell replication for the transgene integration, (3) the relatively labile property of retroviral particles in comparison with other viruses and low titers of up to 10^6 infectious particles per ml, (4) the difficulty in keeping the safely standard from possible biohazard of virus replication and infection, and (5) more seriously the short maximum DNA fragment length to be inserted into the vector, up to 7 kb for retrovirus vectors (Dzau et al., 1993; Friedmann and Jinnah, 1993). The last constraint may also limit an extensive application of the adenoviral vectors as a means of DNA transfer.

The above limitations of the use of viral vectors give an impetus to alternative nonviral means of gene transfer. Chemical or physical LIVGET techniques bear no such limitations with less chances of creating biohazard. In contrast to retroviral vectors, however, integration of transfected genes cannot be usually expected by nonviral LIVGET means. Accordingly, animals thus produced become transfectgenic unless attempts are made to increase integration frequency, resulting in the production of "somatotransgenic" animals. The number of recent papers concerning nonviral gene transfer methods *in vivo* indicates a rapid increase in lipofection and direct DNA injection as reviewed by Muramatsu et al. (1998a). The advantages and disadvantages of these nonviral methods have been described elsewhere (Schofield and Caskey, 1995; Felgner, 1997). By taking account of transfer efficiency, *in vivo* lipofection (Northrop et al., 1987), *in vivo* gene gun (Williams et al., 1991; Zelenin et al., 1997) and *in vivo* electroporation (EP) (Muramatsu et al., 1996a, b, 1997c) may represent convenient and efficient means in general.

Comparison among nonviral gene transfer methods

Direct comparison of nonviral gene transfer methods under *in vivo* conditions has not been documented in the literature except for a few reports (Muramatsu et al,, 1996b, 1997a, c, 1998a). Although such comparison is quite difficult as each method has its own characteristics, researchers often cannot be free from the temptation to ask by themselves which nonviral method is the most efficient. This subsection is devoted to the comparison of nonviral methods.

When three nonviral gene transfer methods were compared in the mouse testis and chicken embryos, *in vivo* (or *in ovo*) electroporation (EP) was more efficient than *in vivo* lipofection or *in vivo* gene gun methods (Muramatsu et al., 1996b, 1997a, c). Figure 2 represents typical examples of foreign gene expression by the three nonviral methods in chicken embryos, indicating the dominance of *in vivo* EP over other methods. In the muscle, direct injection of various genes has been increasingly employed for immunization (Schofield and Caskey, 1995; Felgner, 1997). The efficiency of gene transfer was found to be higher in regenerating muscle and in young animals (Wells and Goldspink, 1992; Davis et al., 1994). Although the mechanism by which cells take up and express purified DNA remains unknown, it may involve the mechanical destruction of the cell membrane. DNA molecules probably penetrate through

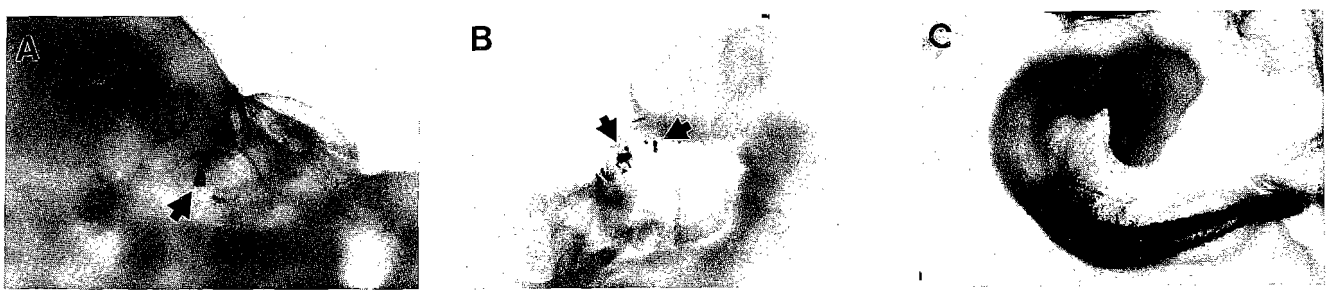

Figure 2. X-gal stained chicken embryos to which the bacterial lacZ gene driven by RSV-LTR and chicken β -actin promoter was transfected *in ovo* by gene gun (A), lipofection (B) or electroporation (C). Detection of the lacZ gene expression by X-gal staining was conducted at 48 hrs after transfection. X-gal stained small spots are indicated by arrow in A and B. (Taken from Muramatsu et al., 1998a).

the damaged cell membrane (Nakanishi, 1995). However, gene expression by this direct injection method is low and generally localized only near the injection sites. In our preliminary experiment with the rat muscle and the mouse skin, *in vivo* EP had more efficient foreign gene expression, approximately 50 times higher than did the direct DNA injection (unpublished results). Recently, Aihara et al. (1998) also reported in the mouse muscle that *in vivo* EP showed enhanced gene expression as high as several hundred to a thousand fold in comparison with direct DNA injection.

In summary, although so far extensive comparison of gene transfer efficiency has not been done among nonviral methods, our data and others in the literature suggest that *in vivo* EP would be one of the best choice as a nonviral gene transfer means.

Regulation of gene expression in transfectgenic animals

A serious concern of the use of transfectgenic animals for therapeutic purposes is whether or not the expression of transferred genes is properly regulated *in vivo* as intended. Because transgenes are basically present in an episomal form, *a priori* assumption is that gene expression diminishes as time goes by. The first obvious question is then how long transgene expression can be maintained. Perhaps, the next question is whether or not transgene expression can be confined to a specific tissue or even to a specific type of cells. The important question is whether or not transgene expression can be induced or repressed as intended. These questions are partially addressed by viewing subsequent several examples.

Duration of gene expression depends on target tissues and to a certain extent, on DNA constructs. In the rat liver, lacZ reporter gene expression was maintained for at least 21 days after transfection, although the expression intensity decreased (Heller et al., 1996). In our preliminary experiment with mice, luciferase gene expression in the liver lasted only for 7 days. Difference in the electrodes used might account for partly, if not entirely, the difference in the duration of gene expression; the former scientists used a hexagonal needle-array electrode whereas in our study a pincette type electrode was used.

If muscles are the target site, the duration of gene expression would be far longer than that in any other tissues. Gene expression after simple DNA injection was maintained in this tissue for several months (Tripathy et al., 1996a, b), and even more than 15 months after *in vivo* EP (unpublished results). Therefore, for gene therapy where long-term gene expression is desired, application of *in vivo* EP to muscles would offer a good chance of supplying therapeutic and physiologically active proteins including hormones that are synthesized in the muscle and secreted into the blood circulation.

In the testis, the duration of gene expression may be longer than that in the liver, but shorter than that in the muscle. LacZ gene expression in the mouse testis was observed at two months after transfection (Yamazaki et al., 1998). Moreover, by using *in vivo* gene gun method, the gene expression in the mouse testis has also been observed at 1 month post-transfection when self-replication sequences of Epstein-Barr virus were included in the DNA construct (Muramatsu et al., 1997b).

Because both viral and nonviral LIVGET are generally conducted at a limited area of a target tissue, the expression of transferred genes is confined therein unless expressed recombinant proteins are secreted into the blood circulation. In this sense, gene expression attained by LIVGET techniques is basically target area-specific even though a universal promoter is used. However, one might often wish to express foreign genes only in a particular type of cells in the limited area of a target tissue. How faithfully is a foreign gene expressed in specific cells after transfection? Although it is premature to conclude whether or not tight regulation of transgene expression is satisfactorily attained, our preliminary results implicate that cell-specific expression is attainable if a proper promoter is chosen. Remarkably stronger CAT gene expression was found in the testis than in the leg muscle or liver of mice by the use of the mouse protamin-1 promoter (Muramatsu et al., 1997a), which was deemed to act predominantly in spermatids as demonstrated in the genuine transgenic mice (Zambrowics et al., 1993).

Over-expression of a foreign gene is not always sufficient for therapeutic purposes: transgene expression should be induced or diminished as intended. Keys to resolve this question may purely lie in promoter constructs. For the inducible gene expression, we used a combination of the chicken oviduct and specific promoters containing steroid response elements, i.e., MMTV-LTR and the chicken ovalbumin promoter. It is well known that gene transcription driven by these promoters is induced by steroid hormones (Sanders and McKnight, 1988; Tsai and O'Malley, 1994; Muramatsu and Sanders, 1995). As was expected, the results indicated that steroid gave induced CAT gene expression driven by the MMTV-LTR and ovalbumin-900 promoters. No induction was found by the SV40 and ovalbumin-100 promoters that are without steroid response elements (Park and Maramatsu, 1999). These findings clearly support the hypothesis that controlled gene expression is possible in transfectgenic animals as found in genuine transgenic animals.

In practice, pharmacological doses of steroid hormone administration as used in our study are not

desirable. Instead, a tetracycline-, or rapamycin-dependent gene expression system could be used for both induction and repression of gene expression (Hulsey et al., 1996). Ye et al. (1999) achieved the delivery of a therapeutic protein under the control of rapamycin through *in vivo* somatic gene transfer. Administration of rapamycin resulted in 200-fold induction of plasma erythropoietin. Alternatively, increasing attention may be paid to nutritional regulation as a milder and more preferable induction system. This possibility has been demonstrated in genuine transgenic mice in the literature (McGrane et al., 1988; Giralt et al., 1991). The same was also true for transfectgenic animals: fasting conferred a similar increase in reporter gene expression in the liver of transfectgenic mice when driven by the promoter of the phosphoenol pyruvate carboxykinase gene, but not by the SV40 promoter (unpublished results).

Applications to production and therapeutics

Few applications of transfectgenic and somato-transgenic animals for therapeutics have been reported. In general, the level of foreign gene expression in these types of transgenic animals is low as compared with that in genuine transgenic animals. However, the most important characteristic of transfectgenic and somatotransgenic animals is the prompt gene expression, being as quick as several hours post-transfection. In contrasts, in genuine transgenic animals the expressed protein cannot be recovered from the mammary gland until adulthood. In cattle, for instance, it takes approximately two years from the genetic manipulation of one-cell embryos till their full development to adult lactating cows. Thus, when quick production of pharmaceutical proteins is the aim, transfectgenic and somatotransgenic animals would be of great value.

By using a retroviral vector, Archer et al. (1994) demonstrated the introduction of human growth hormone (GH) gene in the goat mammary gland, and its successful secretion in the milk from the following day. However, the concentration of human GH secreted into the goat milk was so low that their practical use must await further refinement : a 100- to 1000-fold induction would have to be achieved for efficient protein production.

For gene therapy of humans, somatotransgenic techniques have been discussed in detail in recent reviews (Dube and Cournoyer, 1995; Vile et al., 1996). Most successful applications to gene therapy come from the studies in skin. Originally, *in vivo* transdermal drug and antibody delivery was attempted for various types of cancers in this tissue (Orlowski et al., 1988; Mir et al., 1991; Belehradek et al., 1993; Prausnitz et al., 1993; Marrero et al., 1995). Recently, however, depth-targeted gene delivery into skin was demonstrated in mice (Zhang et al., 1996), which would allow treatment of skin cancers in combination with the use of tumor-suppressor genes such as p53 (Harris et al., 1996; Boulikas, 1997; Hsiao et al., 1997; Nielsen et al., 1997). Brain cancer treatment is another example. In the rat brain tumor, local expression of the human monocyte chemoattractant protein-1 cDNA was transferred, and expressed locally (Nishi et al., 1996). The presence of large numbers of macrophages and lymphocytes observed in the treated tumor tissue indicates a tremendous potential of LIVGET methods for gene therapy of brain cancer.

Manipulations of hormonal status in transfectgenic animals may also offer an important application. It was found in our preliminary experiments that growth of the chicken defective in the growth hormone receptor gene was significantly improved, and plasma IGF-I levels were significantly elevated by *in vivo* transfer of the chicken IGF-I gene (unpublished results). Calculation indicates that if similar growth promotion is to be attained exclusively by the IGF-I protein infusion, the cost would be enormous, being as high as two to three thousand times compared with *in vivo* gene EP treatment. Similarly, rat gastrin levels in plasma were significantly elevated by *in vivo* EP with the rat gastrin cDNA for over 1 month (unpublished results). The *in vivo* introduction of erythropoietin gene resulted in an elevated packed cell volume from 45% to above 70%, the level being maintained for the subsequent several months (Tripathy et al., 1996b; Ye et al., 1999). Such systemic treatments in transfect-genic animals and humans would further expand the possibility of therapeutics manipulations.

GENUINE TRANSGENIC ANIMALS AND THEIR APPLICATIONS

In the following sections, controlled transgene expression and therapeutic applications in "genuine transgenic" animals are discussed. For other aspects of "genuine transgenic" animals, readers are referred to a recent review (Muramatsu and Nakamura, 1997).

Enhanced and tissue-specific transgene expression

1) Tissue-specific expression

Protein-coding genes in higher eukaryotes are mostly transcribed by RNA polymerase Ⅱ. Their promoters resided upstream of the transcription start site, and a number of regulatory elements would determine rigid temporal- and spatial-specificity of gene transcription. For mammary gland-specific expression of foreign genes, for instance, gene promoters derived from α Sl-casein, β-casein, whey acidic protein, α-lactalbumin, and β-lactoglobulin genes have been successfully used. Several hundred to

a few thousand base pairs of these promoter sequences are considered to be the minimal region for the mammary gland-specific expression (Groenen and van der Poel, 1994). However, not all the milk protein gene promoters may confer a high level of recombinant protein production. Neither the 8 kb bovine αS2- nor the 5 kb κ-casein gene promoter sequences gave properly controlled transgene expression (Rijnkels et al., 1995). The reason for the poor expression in these studies remains unknown. All the necessary regulatory elements as well as unknown factors such as higher order DNA structure in these promoter regions may have been lacking.

2) Position-independent expression of the transgenes

It has been recognized that even with the same promoters used, not all transgenic animals express transgenes at a high level as intended. The expression level of transgenes varies from one animal line to another, and is often much lower than would be expected by endogenous genes (Palmiter and Brinster, 1986). Moreover, the gene expression has sometimes been observed in unexpected tissues. Since transgene expression is believed to be largely dependent on the chromosomal site where the gene is integrated (Palmiter and Brinster, 1986), such disturbance in gene expression is termed "position effect". Admittedly, to maintain many transgenic animals with little or no transgene expression increases the total cost. Therefore, surmounting the position effect of integrated transgenes is necessary in practice for efficient recombinant protein production.

Transgenic mice with the 3 kb rat whey acidic protein (WAP) gene showed copy number-dependent and position-independent expression (Krnacik et al., 1995). Similarly, transgenic mice with the 7 kb ovine β-lactoglobulin gene also showed position-independent expression (Webster et al., 1997). However, in both studies ectopic expression was observed with the expression level lower than that of the endogenous β-lactoglobulin gene. Thus, neither the 3 kb region of the WAP gene nor the 7 kb region of β-lactoglobulin gene appeared to have all the essential elements further upstream or downstream for rigid tissue-specific and abundant transgene expression.

To mitigate the position effect, several regulatory sequences have been identified. For example, a locus control region (LCR) was found to confer position-independent and copy number-dependent expression of the human β-globin gene in the transgenic mouse (Grosveld et al., 1987). LCRs are thought to open chromatin structure to facilitate the access of transcription factors (Ellis et al., 1996). Matrix attachment regions (MARs) or scaffold-associated regions (SARs) that are known as attachment sites of chromosomal DNA to nuclear structures (Cockerill and Garrard, 1986; Gasser and Laemmli, 1986) also improved transgene expression (McKnight et al., 1992). In addition, specialized chromatin structures (SCSs) that are possible boundaries of a heat shock gene (Udvardy et al., 1985) allowed enhanced transgene expression (Kcellum and Shedl, 1991).

Besides the inclusion of far upstream or down stream regulatory regions, another strategy to overcome the position effect is to rescue poorly-expressed target genes by co-injecting a high expression vector (McKnight et al., 1995), although this method does not always work (Barash et al., 1996). When the DNA constructs containing factor IX or α-antitrypsin cDNA which usually confers poor expression were microinjected in mouse embryos together with the ovine β-lactoglobulin gene which gives high expression, the two constructs were localized in the same chromosomal position in a ligated form. The results showed that the expression of integrated factor IX and α-antitrypsin genes was substantially improved (Clark et al., 1992). The chromosomal structure around the transgene might have been changed by the highly transcribed β-lactoglobulin gene, resulting in a preferable environment for co-transferred cDNA expression (Yull et al., 1997).

The third and probably the most promising approach to resolve the position effect is the use of yeast artificial chromosome (YAC) as an expression vector of transgenes. The transgenic rats that carry a 210 kb YAC containing the human α-lactalbumin gene expressed the transgene in a position-independent manner (Fujiwara et al., 1997c). When the human GH gene was incorporated into the α-lactalbumin YAC vector, position-independent expression of the hGH gene was also observed (Fujiwara et al., 1999a). The elements necessary for the position-independent expression are considered to reside in the -50 to -20 kb upstream and/or +20 to 50 kb downstream regions of the α-lactalbumin gene (Fujiwara et al., 1999b). Furthermore, transgenic rats harbouring the 210 kb YAC vector in which the human α-lactalbiunin promoter was replaced with the bovine αS1-casein promoter conferred similar position-dependent expression (Fujiwara et al., 1999b). The elements required for the position-independent expression in the bovine α S1-casein gene are somewhat different from those of the human α-lactalbumin gene, despite the fact that the two genes have similar tissue- and developmental stage-specificity. Apparently, it may not be the length of YAC constructs but some regulatory elements that allow high expression of transgenes. A 400 kb casein gene YAC construct (Fujiwara et al., 1997b) failed to express a transgene in the rat (unpublished data). Because casein gene family spans over 400 kb (Fujiwara et al., 1997a), LCRs or other regulatory elements may be present outside of the 400 kb region

in the construct.

Finally, gene targeting technology for STET animal production can be employed to eliminate the position effect of transgene expression. It was considered in the past that the establishment of ES cell lines was indispensable for gene targeting. Apart from mice, however, no available ES cells have been reported in other animal species. Thus, the idea of knock-out and knock-in animals appeared to be elusive. To our surprise, however, the possibility came into the sight again by the recent findings that nuclear transfer technology with cultured cells enabled to obtain viable offspring (Campbell et al., 1996; Wells et al., 1997; Wilmut et al., 1997; Kato et al., 1998; Wakayama et al., 1998), and transgenic sheep, calves and goats (Schniek et al., 1997; Cibelli et al., 1998; Baguisi et al., 1999). In cultured cells, gene targeting can be accomplished satisfactorily. If this is done in donor cells, knock-in animals with respect to milk genes might become available in the future. In knock-in animals where DNA sequences of endogenous milk genes are correctly replaced by transgenes, position-independent expression should be attained in theory.

3) Expression of genomic gene constructs

As Pamiter et al. (1991) have pointed out, genomic constructs in general give higher expression than cDNA counterparts. This has been repeatedly confirmed from the finding that the insertion of introns in cDNA resulted in better gene expression (Brinster et al., 1988; Whitelaw et al., 1991; Hurwitz et al., 1994), although there are a few exceptions (Hurwitz et al., 1994; Korhonen et al., 1997). The reason for the failure of expression or poor expression of cDNA remains unknown, but it may be ascribable to unexpected splicing as found in the factor IX cDNA (Yull et al., 1995) or the presence of silencer as reported in the factor VIII cDNA (Paleyanda et al., 1997).

4) Other approaches

Enhanced and tissue-specific transgene expression could be achieved by approaches other than those described above. First, several drug inducible gene expression systems have been proposed to tightly control transgene expression (Saez et al., 1997). The reverse tetracycline-controlled system was applied for regulating the expression of transgene in the mammary gland (Soulier et al., 1999).

Secondly, tissue-specific expression can be achieved by targeted organ-specific recombination and subsequent activation of gene expression by using the Cre or FLP recombination system (Orban et al., 1992; Ludwig et al., 1996; Vilotte et al., 1998). A recombinase gene was expressed by mammary gland-specific promoters, and foreign gene expression was activated by deleting target sequences located between the promoter and the gene. A more stringent control of the recombinase expression can be achieved by a tetracycline-responsive system (Kistner et al., 1996).

In addition, a large amount of physiologically active recombinant proteins can be produced by intentionally introducing fusion genes that encode physiologically inactive chimeric proteins as a result of conformational changes. The chimeric recombinant proteins would, therefore, little affect the physiology of transgenic animals. Although this approach does not directly aim at enhanced and tissue-specific transgene expression, overall production efficiency may be improved when intact recombinant proteins impose severe detrimental effects on host animals. For example, the production of human erythropoietin in the mammary gland of transgenic mice resulted in severe polycythemie, and hence efficient erythropoietin production was not attainable in these animals (Suk et al., 1995; Uusi-Oukari et al., 1997). This problem was overcome by introducing the β-lactoglobulin/erythro--poietin fusion gene which encodes physiologically inactive chimeric protein (Korhonen et al., 1997). The chimeric proteins may be enzymatically cleaved in the downstream processing later on.

Use for medical and therapeutic purposes

1) Recombinant protein production

Synthesizing and secreting recombinant proteins by transgenic animals are thought to be attractive systems for the production of valuable pharmaceutical proteins. Most studies aim to express genes in mammary gland, although the protein production in some other organs has been reported. As an example of extra-mammary gland expression, functional human hemoglobin was produced in transgenic swine blood (Swanson et al., 1992). Using a bladder specific uroplakin gene promoter, human GH was produced into the urine of transgenic mice (Kerr et al., 1998).

However, these approaches are not practical because much blood cannot be collected without harm to the animals, and protein concentration in urine is too low. Thus, the following description is related exclusively to mammary gland-targeted production.

The mammary gland-targeted transgenic animal bioreactor has several advantages over other production systems including cultures of mammalian cells, bacterial cells or other tissues of transgenic animals as reviewed elsewhere (Houdebine, 1994). A large amount of recombinant proteins can be obtained. For example, a cow can produce over 6,000 L milk a year, containing 30 g/L of protein or more. The secreted proteins are properly processed and modified

posttranslationally (Houdebine, 1994). Moreover, the running cost for rearing animals is much lower than that for maintaining cell culture facilities. Produced recombinant proteins may cause little disturbance with the health of host animals, because recombinant proteins are immediately secreted in the lumen of mammary gland, outside of the body. Down-stream processing is relatively easy to purify the protein from the milk, since it has a simple protein composition (Wilkins and Velander, 1992).

Genes encoding pharmaceutical proteins with milk gene promoters can be introduced into germ lines via microinjection, and expressed specifically in the mammary gland. Although so far many transgenic animals that produce recombinant pharmaceutical proteins in the milk have been reported, a limited number of studies have attained high level expression. Examples of transgenic animals expressing foreign proteins are listed in table 2. Human clinical trials are now being conducted for some recombinant proteins listed in table 2. A large scale production of pharmaceutical proteins by this transgenic system may be brought to realization in the near future.

2) Xenotransplantation

Progress of transplantation therapy in terminal organ failure has resulted in shortage of human donor organs. This has led one to conduct extensive studies in search of alternative organ sources including xenotransplantation in which donor organs from different animal species are utilized. As far as xenograft transplantation is concerned, a suitable donor animal species would be pigs because their organs are almost the same size as those of humans with anatomical and physiological similarities (Cooper et al., 1991). Pigs usually produce many young, and their diseases and rearing technology are well studied. These characteristics make it easy to maintain a large number of healthy donor animals. In addition, little ethical rejection is expected since pigs have been reared for food for many years.

However, organs from animals species like pigs that are discordant from humans cannot be easily transplanted due to severe and prompt rejection (Calne, 1970). When pig organs were transplanted into humans, the transplanted organs were rapidly attacked by the recipient's immune system in an aggressive way. This type of immune response is termed

Table 2. Summary of pharmaceutical proteins produced in the mammary gland of "genuine transgenic" animals

Proteins	Promoters	Transgenic species	Max. Conc. mg/ml	Reference
α-1 antitrypsin	ovine β-lactoglobulin	sheep	35	Wright et al., 11991
Acid α-glucosidase	bovine α S1-casein	mice	1.5	Bijvoet et al., 1996
Antithrombin	goat β casein	goats	3	Edmunds et al., 1998
Bile-salt-stimulated lipase	mouse whey acidic protein	mice	1	Stromqvist et al., 1996
Bovine tracheal AP	mouse whey acidic protein	mice	0.005	Yarus et al., 1996
Erythropoietin	bovine β-lactoglobulin	rabbits	0.5	Korhonen et al., 1997
Factor Ⅷ	mouse wey acidic protein	pigs	0.0027	Paleyanda et al., 1997
Factor Ⅸ	ovine β-lactoglobulin	mice	0.06	Yull et al., 1995
Fibrinogen	ovine β-lactoglobulin	mice	2	Prunkard et al., 1996
Granulocyte-macrophage CSF	bovine α S1-casein	mice	4.6	Uusi-Oukari et al., 1997
Growth hormone	rabbit whey acidic protein	mice	22	Devinoy et al., 1994
Insulin-like growth factor	bovine α S1-casein	rabbits	1	Brem et al., 1994
Interferon-gamma	ovine β-lactoglobulin	mice	0.57	Lagutin et al., 1999
Interleukin-2	rabbit β-casein	mice	0.00043	Buhler et al., 1989
Lactoferrin	bovine α S1-casein	mice	0.036	Patenburg et al., 1994
Lysozyme	bovine α S1-casein	mice	0.28	Maga et al., 1998
Nerve growth factor beta	bovine α S1-casein	rabbits	0.25	Coulibaly et al., 1999
Parathyroid hormone	mouse whey acidic protein	mice	0.000415	Rokkones et al., 1995
Protein C	mouse whey acidic protein	pigs	1	Velander et al., 1992
Procolagen	ovine β-lactoglobulin	mice	0.2	John et al., 1999
Salmon calcitonin	ovine β-lactoglobulin	mice	2.1	McKee et al., 1998
Serum albumin	ovine β-lactoglobulin	mice	2.5	Shani et al., 1992
Superoxide dismutase	mouse whey acidic protein	rabbits	3	Stromqvist et al., 1997
Surfactant protein B	mouse whey acidic protein	mice	5% of WAP mRNA	Yarus et al., 1997
Tissue plasminogen activator	mouse whey acidic protein	goats	3	Ebert et al., 1994
Urokinase	bovine α S1-casein	mice	2	Meade et al., 1990

Abbreviations AP, antimicrobial peptide; CSF, colony-stimulating factor.

hyperacute rejection (Sachs and Bach, 1990). The hyperacute rejection which immediately starts within several minutes to several hours is induced mainly by an antigen-antibody reaction between the donor organ and human natural antibodies with the activation of recipient complement system (Dalmasso et al., 1992; Johnston et al., 1992).

The hyperacute rejection is not suppressed by the currently available immuno-suppressive agents that are being used for chronic treatments. One of the approaches to resolve this problem is the use of inhibitory proteins. Complement system is known to have several such species-specific regulatory proteins to suppress complement activation (RCA) (Takizawa et al., 1992). They include decay accelerating factor (DAF) (Nicholson-Weller et al., 1982), membrane co-factor protein (MCP) (McNearney et al., 1989), and homologous restriction factor (HRF-20 or CD59) (Okada et al., 1989). If pig organs express human RCAs, the transplanted pig organs may not substantially activate human complement system (Dalmasso et al., 1991; Oglesby et al., 1992). This hypothesis was tested by producing the transgenic animals harbouring and expressing human RCA genes in mice (Carry et al., 1993; Diamond et al., 1994), and pigs (Fodor et al., 1994; Rosengard et al., 1995; Langford et al., 1996). Transplanted organs from the transgenic pig expressing human DAF functioned seemingly normally for long periods in primate recipients for up to two months (Lambrigts et al., 1998).

Alternatively, one could possibly diminish the major histocompatibility antigen by diverting the reaction catalyzed by α1,3-galactosyltransferase towards that by α1,2-fucosyltransferase. It is the α1,3-galactosyltransferase in pigs that catalyses the production of the αGal antigen, a cell surface major histocompatibility antigen considered to be responsible for the hyperacute rejection in humans (Cooper et al., 1994; Sandrin et al., 1994). Overexpression of the α1,2-fucosyltransferase, which competes with the α1,3-galactosyltransferase for the same substrate, should result in reduced substrate supply for the latter, and thereby lead to lowered production of the αGal antigen. Indeed, transplanted organs from transgenic mice carrying and expressing the human α1,2-fucosyltransferase showed prolonged survival time in *ex vivo* studies on xenograft rejection (Chen et al., 1998; Cowan et al., 1998).

Despite the above efforts, it may be insufficient to completely suppress xenograft rejection. A theoretically better approach is to produce knock-out pigs with respect to the major histocompatibility antigen gene encoding α 1,3-galactosyltransferase. Because humans do not have an active α1,3-galactosyltransferase gene (Larsen et al., 1990), pig donor organs without this gene cannot evoke the hyperacute rejection. The production of α1,3-galactosyltransferase gene knock-out pigs might be achieved by either ES cell technology or nuclear transfer technology. So far, however, successful production of such knock-out pigs has not yet been reported.

CONCLUSION

In the present review, new nomenclature of various "transgenic" animals and cells was proposed as they can no longer be satisfactorily grouped by classical terms of exclusively either "transgenic" or "nontransgenic". Of these, "transfectgenic and somato-transgenic" and "genuine transgenic" animals were described in detail from a view point of their therapeutic use. The former are useful for rapid production of recombinant proteins in the order of several hours to days, although the level of gene expression is generally low. In contrast, the latter are useful for a large-scale recombinant protein production especially in the mammary gland, and could also serve to supply xenotransplantation organs for human patients. In each case, however, a number of obstacles still exist to accomplish these therapeutic uses in practice. Possible research targets were suggested to surmount the problems encountered at present.

REFERENCES

Aihara, H. and J. Miyazaki. 1998. Gene transfer into muscle by electroporation *in vivo*. Nat. Biotechnol. 16:867-70.

Archer, J. S., W S. Kennan, M. N. Gould and R. D. Bremel. 1994. Human growth hormone (hGH) secretion in milk of goats after direct transfer of the hGH gene into the mammary gland by using replication-defective retrovirus vectors. Proc. Natl. Acad. Sci. USA, 91: 6840-6844.

Baguisi, A., E. Beboodi, D. T. Melican, J. S. Pollock, M. M. Destrempes, C. Cammuso, J. L. Williams, S. D. Nims, C. A. Porter, P. Midura, M. J. Palacios, S. L. Ayers, R. S. Denniston, M. L. Hayes, C. A. Ziomek, H. M. Meade, R. A. Godke, W. G. Gavin, E. R. Overstrom and Y. Echelard. 1999. Production of goats by somatic cell nuclear transfer. Nat. Biotechnol. 17:456-461.

Barash, I., N. Ilan, R. Kari, D. R. Hurwitz and M. Shani 1996. Co-integration of β-lactoglobulin/human serum albumin hybrid genes with the entire β-lactoglobulin gene or the matrix attachment region element: repression of human serum albumin and β-lactoglobulin expression in the mammary gland and dual regulation of the transgenes. Mol. Reprod. Dev. 45:421-430.

Belehradek, M., C. Domenge, B. Luboinski, S. Orlowski, J. Belehradek, Jr. and L. M. Mir. 1993. Electrochemotherapy, a new antitumor treatment. First clinical phase I-II trial. Cancer. 72:3694-3700.

Bijvoet, A. G. A., M. A. Kros, F. R. Pieper, H. A. de Boer, A. J. J. Reuser, A. T. der Ploeg and M. P. Verbeet. 1996. Expression of cDNA-encoded human acid

α-glucosidase in milk of transgenic mice. Biochim. Biophys. Acta. 1308:93-96.

Boulikas, T. 1997. Gene therapy of prostate cancer: p53, suicidal genes, and other targets. Anticancer Res. 17: 1471-1505.

Brem, G., P. Hartl, U. Besenbfelder, E. Wolf and N. Zinovieva. 1994. Expression of synthetic cDNA sequences encoding human insulin-like growth factor-I (IGF-I) in the mammary gland of transgenic rabbits. Gene. 149:351-355.

Brinster, R. L., J. M. Allen, R. R. Behringer, R. E. Gelinas and R. D. Palmiter. 1988. Introns increase transcriptional efficiency in transgenic mice. Proc. Natl. Acad. Sci. USA. 85:836-840.

Buhler, T. A, T. Bruyere, D. F. Went, G. Stranzinger and K. Burki. 1989. Rabbit β-casein promoter directs secretion of human interleukin-2 into the milk of transgenic rabbits. Biotechnology. 8:140-144.

Calne, R. Y. 1970. Organ transplantation between widely disparate species. Transplant. Proc. 2:550-553.

Campbell, K. H. S., J. McWhir, W. A. Ritchie and I. Wilmut. 1996. Sheep cloned by nuclear transfer from a cultured cell line. Nature. 380:64-66.

Carry, N., J. Moody. N. Yannoutsos, J. Wallwork and D. White. 1993. Tissue expression of human decay accelerating factor, a regulator of complement activation expressed in mice: a potential approach to inhibition of hyperacute xenograft rejection. Transplant. Proc. 25:400-401.

Chen C. -G, E. J. Salvaris, M. Romanella, A. Aminian, M. Katerelos, N. Fisicaro, A. J. F. D'Apice, and M. J. Pearse. 1998. Transgenic expression of human of α1,2-fucosyltransferase (H-transferase) prolongs mouse heart survival in an *ex vivo* model of xenograft rejection. Transplantation. 65:832-837.

Chong, H. and R. G. Vile. 1996. New therapeutic approaches based on gene trangfer technologies. Springer. Semin. Immunopathol. 18:149-170.

Cibelli, J. B., S. L. Stice, P. J. Golueke, J. J. Kane, J. Jerry, C. Blackwell, F. A. Ponce de Leon and J. M. Robl. 1998. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science. 280:1256-1258.

Clark, A. J., A. Cowper, R. Wallace, G. Wright and J. P. Simons. 1992. Rescuing transgene expression by co-injection. Bio/Technology. 10:1450-1454.

Cockerill, P. N. and W. T. Garrard. 1986. Chromosomal loop anchorage of the kappa immunogloblin gene occurs next to the enhancer in a region containing topoisomerase II site. Cell. 44:273-282

Cooper, D. K. C., E. Koren and R. Oriol. 1994. Oligosaccharides and discordant xenotransplantation. Immunol. Rev. 141:31-58.

Cooper, D. K. C., Y. Ye, L. L. Rolf and N. Zuhdi. 1991. The pig as potential organ donor for man. In: Xenotransplantation. Cooper, D. K. C., E. Kemp, K. Reemtsma and D. J. G. White eds. Springer-Verlag, pp. 481-500.

Coulibaly, S., U. Besenfelder, M. Fleischmann, N. Zinovieva, A. Grossmann, M. Wozny, I. Bartke, R. Toegel, M. Muller and G. Brem. 1999. Human nerve growth factor beta (hNGF-b): mammary gland specific expression and production in transgenic rabbits. FEBS Lett. 444:111-116.

Cowan P. J., C.-G. Chen, T. A. Shinkel, N. Fiscaro, E. Salvaris, A. Aminian, M. Romanella, M. J. Pearse, and A. J. F. D'Apice. 1998. Knock out of α1,3-galactosyltransferase or expression of α1,2-fucosyltransferase further protects CD55- and CD59-expressing mouse hearts in an ex vivo model of xenograft rejection. Transplantation. 65:1599-1604.

Dalmasso, A. P., G. M. Vercellotti, R. J. Fiscehel, R. M. Bolman, F. H. Bach and J. L. Platt. 1992. Mechanism of complement activation in the hyperacute rejection of porcine organs transplanted into primate recipients. Am. J. Pathol. 140:1157-1166.

Dalmasso, A. P., G. M. Vercellotti, J. L. Platt and F. H. Bach. 1991. Inhibition of complement-mediated endothelial cell cytotoxicity by decay-acceleration factor. Transplantation. 52:530-533.

Davis, H. L., M. L. Michel, M. Mancini, M. Schleef and R. G. Whalen. 1994. Direct gene transfer in skeletal muscle: plasmid DNA-based immunization against the hepatitis B virus surface antigen. Vaccine. 12:1503-1509.

Devinoy, E., D. Thepot, M. G. Stinnakre, M. L. Fontaine, H. Grabowski, C. Puissant, A. Pavirani and L. M. Houdebine. 1994. High level production of human growth hormone in the milk of transgenic mice: the upstream region of the rabbit whey acidic protein (WAP) gene targets transgene expression to the mammary gland. Transgenic Res. 3:79-89.

Diamond, L. E., E. R. Oldham, J. L. Waldmann, M. Tone, L. A. Walsh and J. S. Logan. 1994. Cell- and tissue-specific expression of a human CD59 minigene in transgenic mice. Transplant. Proc. 26:1239.

Dube, I. D. and D. Cournoyer. 1995. Gene therapy:here to stay. Can. Med. Assoc. J. 152:1605-1613.

Dzau, V. J., R. Morishita and G. H. Bibbons. 1993. Gene therapy for cardiovascular disease. Trends Biotechnol. 11: 205-210.

Ebert, K. M., P. DiTullio, C. A. Barry, J. E. Schindler, S. I. Ayres, T. E. Smith, L. J. Pellerin, H. M. Meade, J. Denman and B. Roberts. 1994. Induction of human tissue plasminogen activator in the mammary gland of transgenic goats. Bio/Technology. 12:699-702.

Edmunds, T., S. M. V. Patten, J. Pollock, E. Hangon, R. Bernasconi, E. Higgins, P. Manavalan, C. Ziomek, H. Meade, J, M. McPherson and E. S. Cole. 1998. Transgenically produced human antithrombin: Structural and functional comparison to human plasma-derived antithrombin. Blood. 91:4561-4571.

Ellis, J., K. C. Tan-Un, A. Harper, D. Michalovich, N. Yannoutsos, S. Philipsen and F. Grosveld. 1996. A dominant chromatin-opening activity in 5' hypersensitive site 3 of human β-globin locus control region. EMBO. J. 15:562-568.

Felgner, P. L. 1997. Nonviral strategies for gene therapy. Sci. Am. 276:102-106.

Fodor, W. L., B. L. Williams, L. A. Matis, J. A. Madri, S. A. Rollins, J. W. Knight, W. Velander and S. P. Squinto. 1994. Expression of a functional human complement inhibitor in a transgenic pig as a model for the prevention of xenogeneic hyperacute organ rejection. Proc. Natl. Acad. Sci., USA. 91:11153-11157.

Friedmann, T. and H. A. Jinnah. 1993. Gene therapy for disorders of the nervous system. Trends Biotechnol. 11:

192-197.

Fujiwara, Y., M. Miwa, M. Nogami, K. Okumura, T. Nobori, T. Suzuki and M. Ueda. 1997a. Genomic organization and chromosomal localization of the human casein gene family. Human Genet. 99:368-373.

Fujiwara, Y., M. Miwa, R. Takahashi, M. Hirabayashi, T. Suzuki and M. Ueda. 1997b. Transgenic animals with various YAC constructs: Preparation of 400 kb YAC DNA for microinjection. J. Reprod. Dev. 43:81-90.

Fujiwara, Y., M. Miwa, R. Takahashi, M. Hirabayashi, T. Suzuki and M. Ueda. 1997c. Position-independent and high-level expression of human α-lactoglobumin in the milk of transgenic rats carrying a 210-kb YAC DNA. Mol. Reprod. Dev. 47:157-163.

Fujiwara, Y., M. Miwa, R. Takahashi, M. Kameda, K. Kodaira, M. Hirabayashi, T. Suzuki and M. Ueda. 1999b. Analysis of control elements for position-independent expression of human α-lactalbumin YAC. Mol. Reprod. Dev. 54:17-23.

Fujiwara, Y., M. Miwa, R. Takahashi, K. Kodaira, M. Hirabayashi, T Suzuki and M. Ueda. 1999a. High-level expressing YAC vector for transgenic animal bioreactor. Mol. Reprod. Dev. 52:414-420.

Gasser, S. M. and U. K. Laemmli. 1986. Cohabitation of scaffold binding regions with upstream/enhancer elements of three developmentally regulated genes of D. melanogaster. Cell. 46:521-530.

Giralt, M., A. E. Park, J. Liu, A. Gurney and R. W. Hanson. 1991. Identification of a thyroid hormone response element in P-enolpyruvate carboxykinase (GTP) gene: evidence for a synergistic interaction between thyroid hormone and cAMP cis regulatory elements. J. Biol. Chem. 266:21991-21996.

Groenen, M. A. M. and J. J. van der Pool. 1994. Regulation of expression of milk protein genes: a review. Livest. Prod. Sci. 38:61-78.

Grosveld, F., G. B. van Assendelft, D. Greaves and G. Kollias. 1987. Position-independent, high-level expression of the human β-globin gene in transgenic mice. Cell. 51:975-985.

Harris, M. P., S. Sutjipto, K. N. Wills, W. Hancock, D. Cornell, D. E. Johnson, R. J. Gregory, H. M. Shepard and D. C. Maneval. 1996. Adenovirus-mediated p53 gene transfer inhibits growth of human tumor cells expressing mutant p53 protein. Cancer Gene Therapy. 3:121-130.

Heller, R., M. Jaroszeski, A. Atkin, D. Moradpour, R. Gilbert, J. Wands and C. Nicolau. 1996. *In vivo* gene electroinjection and expression in rat liver. FEBS Lett. 389:225-228.

Houdebine, L. M. 1994. Production of pharmaceutical proteins from transgenic animals. J. Biotech. 34:269-287.

Hsiao, M., V. Tse. J. Carmel, Y. Tsai, P. L. Felgner, M. Haas and G. D. Silverberg. 1997. Intracavitary liposome-mediated p53 gene transfer into glioblastoma with endogenous wild-type p53 *in vivo* results in tumor suppression and long-term suvival. Biochem. Biophys. Res. Commun. 233:359-364.

Hulsey, M. G., R. J. Martin and C. A. Baile. 1996. The role of transgenic models in nutrition research. In : Gene Expression and Nutrition : From Cells to Whole- Body. Muramatsu, T.ed., Reserach Signpost, Trivandrum, India. pp. 103-123.

Hurwitz, D., M. Mathan, I. Barash, N. Ilan and M. Shani. 1994. Specific combinations of human serum albumin introns direct high level expression of albumin in transfected COS cells and in the milk of transgenic mice. Transgenic Res. 3:365-375.

John, D. C. A, R. Watson, A. J. Kind, A. R. Scott, K. E. Kadler and N. J. Bulleid. 1999. Expression of an engineered form of recombinant procollagen in mouse milk. Nature Biotech. 17:385-389.

Johnston, P. S., M. W. Wang, S. M. L. Lim, L. J. Wright and D. J. G. White. 1992. Discordant xenograft rejection in an antibody free model. Transplantation. 54:573-576.

Kato Y., T. Tani, Y. Sotomaru, K. Kurokawa, J. Kato, H. Doguchi, H. Yasue and Y. Tsunoda. 1998. Eight calves cloned from somatic cells of a single adult. Science. 282: 2095-2098.

Kellum, R. and P. Shedl. 1991. A position-effect assay for boundaries of higher order chromosomal domains. Cell. 64:941-950.

Kerr, D. E., F. Liang, K. R. Bondioli, H. Zhao, G. Kreibich, R. J. Wall and T. T. Sun. 1998. The bladder as a bioreactor: urothelium production and secretion of growth hormone into urine. Nature Biotech. 16:75-79.

Kistner, T., M. Gossen, F. Zimmermann, J. Jerecic, C. Ullmer, H. Lubbert and H. Bujard. 1996. Doxycyclin-mediated quantitative and tissue-specific control of gene expression in transgenic mice. Proc. Natl. Acad. Sci., USA. 93:10933-10938.

Korhonen, V. P., M. Tolvanen, J. M. Hyttinen, M. Uusi-Oukari, R. Sinervirta, L. Alhonen, M. Jauhiainen, O. A. Janne and J. Janne. 1997. Expression of bovine β-lactoglobulin/human erythropoietin fusion protein in the milk of transgenic mice and rabbits. Eur. J. Biochem. 245:482-489.

Krnacik, M. J., S. Li, J. Liao and J. M. Rosen. 1995. Position-independent expression of whey acidic protein tansgenes. J. Biol. Chem. 270:11119-11129.

Lagutin, O. V., V. N. Dobrovolsky, T. V. Vinogradova, B. N. Kyndiakov, Y. M. Khodarovich, N. Jenkins, D. James, N. Markham and O. A. Larionov. 1999. Efficient human IFN-γ expression in the mammary gland of transgenic mice. J. Interferon Cytosine Res. 19:137-144.

Lambrigts D., D. H. Sachs and D. K. C. Cooper. 1998. Discordant organ xenotransplantation in primates. Transplantation. 66:547-561.

Langford, G. A. E. Cozzi, N. Yannoutsos, R. Lancaster, K. Elsome, P. Chen and D. J. G. White. 1996. Production of pigs transgenic for human regulators of complement activation using YAC technology. Transplant. Proc. 28: 862-863.

Larsen, R. D., C. A. Rivers-Marrero, L. K. Ernst, R. D. Cummings and J. B. Lower. 1990. Frameshift and nonsense mutations in a human genomic sequence hormologous to a murine UDP-Gal:β-D-Gal(1,4)-D-GlcNAc α(1,3)-galactosyltransferase. J. Biol. Chem. 265: 7055-7061.

Ludwig, D. L., J. R. Stringer, D. C. Wight, T. C. Doetschman and J. J. Duffy. 1996. FLP-mediated site-specific recombination in microinjected murine zygotes. Transgenic Res. 5:385-395.

Maga, E. A., G. B. Anderson and J. D. Murray. 1995. The effect of mammary gland expression of human lysozyme

on the properties of milk from transgenic mice. J. Dairy Sci. 78:2645-2652.

Marrero, M. B., B. Schieffer, W. G. Paxton, E. Schieffer and K. E. Bernstein. 1995. Electroporation of pp60c-src antibodies inhibits the angiotensin II activation of phospholipase C-gamma 1 in rat aortic smooth muscle cells. J. Biol. Chem. 270:15734-15738.

McGrane, M. M., J. de Vente, J. Yun, J, Bloom, E. A. Park, A. Wynshaw-Boris, T. Wagner, F. M. Rottman and R. W. Hanson. 1988. Tissue-specific expression and dietary regulation of a chimeric PEPCK/bGH gene in transgenic mice. J. Biol. Chem. 263:11443-11451.

McKee, C., A. Gibson, M. Dalrymple, L. Emslie, I. Garner and I. Cottingham. 1998. Production of biologically active salmon calcitonin in the milk of transgenic rabbits. Nature Biotech. 16:647-651.

McKnight, R. A., A. Shamay, L. Sankaran, R. J. Wall and L. Hennighausen. 1992. Matrix-attachment regions can impart position-independent regulation of a tissue-specific gene in trassgenic mice. Proc. Natl. Acad. Sci. USA. 89:6943-6947.

McNearney, T., L. Ballard, T. Seya and J. P. Atkinson. 1989. Membrane cofactor protein of complement is present on human fibroblast, epithelial, and endothelial cells. J. Clin. Invest. 84:538-545.

McKnight, R. A., R. J. Wall and L. Hennighausen. 1995. Expression of genomic and cDNA, transgenes after co-integration in transgenic mice. Transgenic Res. 4:39-43.

Meade, H., L. Gates, E. Lacy and N. Lonberg. 1990. Bovine alpha S_1-casein gene sequences direct high level expression of active human urokinase in mouse milk. Bio/Technology. 8:443-446.

Mir, L. M., S. Orlowski, J. Belehradek Jr. and C. Paoletti. 1991. Electrochemotherapy potentiation of antitumour effect of bleomycin by local electric pulses. Eur. J. Cancer. 27:68-72.

Mitani, K. and C. T. Caskey. 1993. Delivering therapeutic genes - matching approach and application. Trends Biotechnol. 11:162-166.

Mulligan. R. C. 1993. The basic science of gene therapy. Science. 260:926-932.

Muramatsu, T., Y. Mizutani and J. Okumura. 1996a. Live detection of firefly luciferase gene expression by bioluminescence in incubating chicken embryos. Anim. Sci. Technol. Jpn. 67:906-909.

Muramatsu, T., Y. Mizutani, Y. Ohmori and J. Okumura. 1997c. Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos *in ovo*. Biochem. Biophys. Res. Commun. 230:376-380.

Muramatsu, T. and A. Nakamura. 1997. Production of transgenic cattle as an animal bioreactor : current status and perspective. Trends Comp. Biochem. Physiol. 3:9-21.

Muramatsu, T., A. Nakamura and H. -M. Park. 1998a. *In vivo* electroporation : a powerful and convenient means of nonviral gene transfer to tissues of living animals. Int. J. Mol. Med. 1:55-62.

Muramatsu, T., A. Nakamura and H. -M. Park. 1998b. Basics and applications of gene transfer in animal cells and tissues for production and therapeutics. J. Mainm. Ova Res. 15:1-16.

Muramatsu, T. and M. M. Sanders. 1995. Regulation of ovalbumin gene expression. Poult. Avian Biol. Rev. 6: 107-123.

Muramatsu, T., O. Shibata, Y. Ohmori and J. Okumura. 1996b. *In vivo* electroporation : a convenient method for gene transfer to testicular cells in mice. Anim. Sci. Technol Jpn. 67:975-982.

Muramatsu, T., O. Shibata and J. Okumura. 1997b. Effect of self-replication DNA sequences of Epstein-Barr virus on the expression of a foreign gene transfected *in vivo* to the mouse testis. Anim. Sci. Technol. Jpn. 68:650-654.

Muramatsu, T., O. Shibata, S. Ryoki, Y. Ohmori and J. Okumura. 1997a. Foreign gene expression in the mouse testis by localized in vivo gene transfer. Biochem. Biophys. Res. Commu. 233:45-49.

Nakanishi, M. 1995. Gene introduction into animal tissues. Crit. Rev. Ther. Drug Carrier Syst. 12:263-310.

Nicholson-Weller, A., J. Burge, D. T. Fearon, P. F. Weller and K. F. Austin. 1982. Isolation of a human erythrocyte membrane glycoprotein with decay-accelerating activity for C3 convertases of the complement system. J. Immunol. 129:184-189.

Nielsen, L. L., J. Dell, E. Maxwell, L. Armstrong, D. Maneval and J. J. Catino. 1997. Efficacy of p53 adenovirus-mediated gene therapy against human breast cancer xenografts. Cancer Gene Ther. 4:129-138.

Nishi, T., K. Yoshizato, S. Yamashiro, H. Takeshima, K. Sato, K. Hamada, I. Kitamura, T. Yoshimura, H. Saya, J. Kuratsu and Y. Ushio. 1996. High- efficiency *in vivo* gene transfer using intraarterial plasmid DNA injection following *in vivo* electroporation. Cancer Res. 56:1050-1055.

Northrop, J. P, G. M. Ringlod and M. Danielsen. 1987. Lipofection: a highly efficient lipid-mediated DNA- transfection procedure. Proc. Natl. Acad. Sci., USA. 84:7413-7417.

Oglesby, T. J., C. J. Allen, M. K. Liszewski, D. J. G. White and J. P. Atkinson. 1992. Membrane cofactor protein (CD46) protects cells from complement-mediated attack by an intrinsic mechanism. J. Exp. Med. 175: 1547-1551.

Okada, N., R. Harada, T. Fujita and H. Okada. 1989. A novel membrane glycoprotein capable of inhibiting membrane attack by homologous complement. Int. Immunol. 1:205-208.

Orban, P. C., D. Chui and D. Marth. 1992. Tissue- and site-specific DNA recombination in transgenic mice. Proc. Natl. Acad. Sci., USA. 89:6861-6865.

Orlowski, S., J. Belehradek, Jr., C. Paoletti and L. M. Mir. 1988. Transient electropermeabilization of cells in culture. Increase of the cytotoxicity of anticancer drugs. Biochem. Pharmacol. 37:4727-4733.

Paleyanda, R. K., W. H. Velander, T. K. Lee, D. H. Scandella, F. C. Gwazdauskas, J. W. Knight, L. W. Hoyer, W. N. Drohan and H. Lubon. 1997. Transgenic pigs produce functional human factor VIII in milk. Nat. Biotech. 15:971-975.

Palmiter, R. D. and R. L. Brinster. 1986. Germ-line transformation of mice. Ann. Rev. Genet. 20:465-499.

Palmiter, R. D., E. P. Sandgren, M. R. Avarbock, D. D. Allen and R. L. Brinster. 1991. Heterologous intron can enhance expression of transgenes in mice. Proc. Natl. Acad. Sci., USA. 88:478-482.

Park, H. -M. and T. Muramatsu. 1999. *In vivo* manipulation of foreign gene expression by steroid administration in the oviduct of laying hens. J. Endocrionol. (In press).

Platenburg, G. J., E. P. A. Kootijk, P. M. Kooiman, S. L. Woloshuk, J. H. Nuijens, P. J. A. Krimpenfort, F. R. Pieper. H. A. de Boer and R. Strijker. 1994. Expression of lactoferrin in milk of transgenic mice. Transgenic Res. 3:99-108.

Prausnitz, M. R., V. G. Bose, R. Lulger and J. C. Weaver. 1993. Electroporation of mammalian skin : a mechanism to enhance transdermal drug delivery. Proc. Natl. Acad. Sci., USA. 90:10504-10508.

Prunkard, D., I. Cottingham, I. Gamer, S. Bruce, M. Dalrymple, G. Lasser, P. Bishop and D. Foster. 1996. High-level expression of recombinant human fibrinogen in the milk of transgenic mice. Nat. Biotechnol. 14:867-871.

Rijnkels, M., P. M. Kooiman, P. J. A. Krinmpenfort, H. A. de Boer and F. R. Pieper. 1995, Expression analysis of the individual bovine β, αs2- and κ-casein genes in transgenic mice. Biochem. J. 311:929-937.

Rokkones, E., S. H. Fromm, B. N. Kareem, H. Klungland, O. K. Olstad, A. Hogset, J. Iversen. K. Bjoro and K. M. Gautvik. 1995. Human parathyroid hormone as a secretory peptide in milk of transgenic mice. J. Cell Biochem. 59:168-176.

Rosengard, A. M., N. R. B. Carry, G. A. Langford, A. W. Tucker, J. Wallwork and D. J. G. White. 1995. Tissue expression of human complement inhibitor, decay-accelerating factor, in transgeuic pigs. Transplantation. 59: 1325-1333.

Sachs, D. H. and F. H. Bach. 1990. Immunology of xenograft rejection. Hum. Immunol. 28:245-251.

Saez, E. D. N. A. West and R. M. Evans. 1997. Inducible gene expression in mammalian cells and transgenic mice. Curr. Opin. Biotech. 8:608-616.

Sanders, M. M. and G. S. McKnight. 1988. Positive and negative regulatory elements control the steroid-responsive ovalbumin promoter. Biochemistry. 27:6550-6557.

Sandrin, M. S., H. A. Vaughan and I. F. C. McKenzie. 1994. Identification of gal(α1,3)gal as the major epitope for pig-to-human vascularized xenografts. Transplant. Rev. 8:134-149.

Schnieke, A. E., A. J. Kind, W. A. Ritchie, K. Mycock, A. R. Scott, M. Ritchie, I. Wilmut, A. Colman and K. H. Campbell. 1997. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science. 278:2130-2133.

Schofield, J. P. and C. T. Caskey. 1995. Non-viral approaches to gene therapy. Br. Med. Bull. 51:56-71.

Shani, M., I, Barash, M. Nathan, G. Ricca, G. H. Searfoss, I. Dekel, A. Faerman, D. Givol and D. R. Hurwitz. 1992. Expression of human albumin in the milk of transgenic mice. Transgenic Res. 1:195-208.

Soulier, S., M. -G. Stinnakre, L. Lepourry, J. -C. Mercier and J. -L. Vilotte. 1999. Use of doxycycline-controled gene expression to reversibly alter milk-protein composition in transgenic mice. Eur. J. Biochem. 260:533-539.

Stromqvist, M., L. M. Houdebine, J. O. Andersson, A. Edlund, T. Johansson, C. Viglietta, C. Puissant and L. Hansson. 1997. Recombinant human extracellular superoxide dismutase produced in milk of transgenic rabbits. Transgenic Res. 6:271-278.

Stromqvist M., J. Tornell. M. Edlund, A. Edlund, T. Hohansson, K. Lindgren, L. Lundberg and L. Hannson. 1996. Recombinant human bile-salt-stimulated lipase: an example of defective O-glycosylation of a protein produced in milk of transgenic mice. Transgenic Res. 5: 475-485.

Suk, K., D. Y. Jung, S. K. Kang, S. W. Kang, E. J. Seo, H. A. Kand, M. H. Yu and S. J. Seo. 1995. Human erythropoietin-induced polycythemia in transgenic mice. Mol. Cells. 5:634-640.

Swanson, M. E., M. J. Martin, J. K. O'Dounell, K. Hoover, W. Lago, V. Huntress, C. T. Parsons, C. A. Pinkert, S. Pilder and J. S. Logan. 1992. Production of functional human hemoglobin in transgenic swine. Bio/Technology 10:557-559.

Takizawa, H., K. Takahashi, T. Murakami, N. Okada and H. Okada. 1992. Species-specific restriction of complement by HRF20(CD59) generated by cDNA transfection. Eur. J. Immunol. 22:1943-1946.

Tripathy, S, K., H. B. Black, E. Goldwasser and J. M. Leiden. 1996a. Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors. Nat. Med. 2:545-550.

Tripathy, S. K., E. C. Svensson, H. B. Black, E. Goldwasser, M. Margalith, P. M. Hobart and J. M. Leiden. 1996b. Long-term expression of erythropoietin in the systemic circulation of mice after intramuscular injection of a plasmid DNA vector. Proc. Natl. Acad. Sci., USA. 93:10876-10880.

Tsai, M. -J. and B. W. O'Malley. 1994. Molecular mechanisms of action of steroid/thyroid receptor superfimily members. Ann. Rev. Biochem. 63:451-496.

Udvardy, A., E. Maine and P. Schedl. 1985. The 87A7 chromomere. Identification of novel chromatin structures flanking the heat shock locus that may define the boundaries of higher order domains. J. Mol. Biol. 185: 341-358.

Uusi-Oukari, M., J. M. Hyttinen, V. P. Korhonen, A. Vasti, L. Alhonen, O. Janne and J. Janne. 1997. Bovine α s1-casein gene sequences direct high level expression of human granulocyte-macrophage colony-stimulating factor in the milk of tansgenic mice. Transgenic Res. 6:75-84.

Velander, W., J. L. Johnson, R. L. Page, C. G. Russell, A. Subramanian, T. D. Wilkins, F. C. Gwazdauskas, C. Pittius and W. N. Drohan. 1992. High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. Proc. Natl. Acad. Sci., USA. 89:12003-12007.

Vile, R. G., A. Tsuzynski and S. Castleden. 1996. Retroviral vectors. From laboratory tools to molecular medicine. Mol. Biotechnol. 5:139-158.

Vilotte, J. -L., F. L'Huillier and J. -C. Mercier. 1998. Modification and repression of genes expressed in the mammary gland using gene targeting and other technologies. J. Mamm. Gland Biol. Neopl. 3:351-362.

Wakayama, T., A. C. F. Perry, M. Zuccotti, K. R. Johnson and R. Yanagimachi. 1998. Full-term development of mice from enucleated oocytes injected with cumulus cells nuclei. Nature. 394:369-374.

Webster, J., G. Donofrio, R. Wallace, A. J. Clark and C. B. A. Whitelaw. 1997. Intronic sequences modulate the sensitivity of β-lactoglobulin transgenes to position effects. Gene. 193:239-243.

Wells, D. J. and G. Goldspink. 1992. Age and sex influence expression of plasmid DNA directly injected into mouse skeletal muscle. FEBS Lett. 306:203-205.

Werls, D. N., P. N. Misica, T. A. Day and H. R. Tervit 1997. Production of cloned lambs from an established embryonic cell line: a comparison between *in vivo*- and *in vitro*-matured cytoplasts. Biol. Reprod. 57:385-393.

Whitelaw, C. B. A., A. L. Archibald, S. Harris, M. McClenaghan, J. P. Simons and A. J. Clark. 1991. Targeting expression to the mammary gland: intronic sequences can enhance the efficiency of gene expression in transgenic mice. Transgenic Res. 1:3-13.

Wilkins, T. D. and W. Velander. 1992. Isolation of recombinant proteins from milk. J. Cell Biochem. 49: 333-338.

Williams, R. S., S. A. Johnston, M. Reidy, M. J. de Vit, S. G. McElligott and J. C. Sanford. 1991. Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles. Proc. Natl. Acad. Sci., USA. 88:2726-2730.

Wilmut, I., A. E. Schnieke, J. McWhir, A. J. Kind and K. H. Campbell. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature. 385:810-813.

Wright, G., A. Carver, D. Cotton, D. Reeves, A. Scott, P. Simons, I. Wilmut, I. Garner and A. Colman. 1991. High level expression of active human alpha-1-antitrypsin in the milk of transgenic sheep. Bio/Technology. 9:830-834.

Yamazaki, Y., H. Fujimoto, H. Ando, T. Ohyama, Y. Hirota and T. Noce. 1998. *In vivo* gene transfer to mouse spermatogenic cells by deoxyribonucleic acid injection into seminiferous tubules and subsequent electroporation. Biol. Reprod. 59:1439-1444.

Yarus, S., N. M. Greenberg, Y. Wei, J. A. Whitsett, T. E. Weaver and J. M. Rosen. 1997. Secretion of unprocessed human surfactant protein B in milk of transgenic mice Transgenic Res. 6:51-57.

Yarus, S., J. M. Rosen, A. M. Cole and G. Diamond. 1996. Production of active bovine tracheal antimicrobial peptide in milk of transgenic mice. Proc. Natl. Acad. Sci., USA. 93:14118-14121.

Ye, X., V. M. Rivera, P. Zoltick, F. Cerasoli, Jr., M. A. Schnell, G. Gao, J. V. Hughes, M. Gilman and J. M. Wilson. 1999. Regulated delivery of therapeutic proteins after *in vivo* somatic cell gene transfer. Science. 283: 88-91.

Yull, F., B. Binas, G. Harold, R. Wallace and A. J. Clark. 1997. Transgenic rescue in the mammary gland is associated with transcription but does not require translation of BLG transgenes. Transgenic Res. 6:11-17.

Yull, F., G. Harold. R. Wallace, A. Cowper, J. Percy, I. Cottingham and A. J. Clark. 1995. Fixing human factor IX (fIX): correction of a cryptic RNA splice enables the production of biologically active fIX in the mammary gland of transgenic mice. Proc. Natl. Acad. Sci., USA. 92:10899-10903.

Zambrowics, B. P., C. J. Harendza, J. W. Zimmermann, R. L. Brinster and R. D. Palmiter. 1993. Analysis of the mouse protamine 1 promoter in transgenic mice. Proc. Natl. Acad. Sci., USA. 90:5071-5075.

Zhang, L., L. Li, G. A. Hofmann and R. M. Hoffman. 1996. Depth-targeted efficient gene delivery and expression in the skin by pulsed electric fields: an approach to gene therapy of skin aging and other diseases. Biochem. Biophys. Res. Common. 220:633-636.

Zelenin, A. V., V. A. Kolesnikov, O. A. Tarasenko, R. A. Shafei, I. A. Zelenina, V. V. Mikhailov, M. L. Semenova, D. V. Kovalenko, O. V. Artemyeva, T. E. Ivaschenko, O. V. Evgafov, G. Dlickson and V. S. Baranovand. 1997. Bacterial beta-galactosidase and human dystrophin genes are expressed in mouse skeletal muscle fibers after ballistic transfection. FEBS Lett. 414:319-322.