Transgenic Research **9:** 305–320, 2000.

Transgenic animal bioreactors

Louis Marie Houdebine
Unite de Biologie du Développement et Biotechnologie, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas Cedex, France, Tél.: 33 1 34 65 25 40; Fax: 33 1 34 65 22 41; E-mail: houdebine@biotec.jouy.inra.fr

Key words: Recombinant proteins, transgenic animals, milk

Abstract

The production of recombinant proteins is one of the major successes of biotechnology. Animal cells are required to synthesize proteins with the appropriate post-translational modifications. Transgenic animals are being used for this purpose. Milk, egg white, blood, urine, seminal plasma and silk worm cocoon from transgenic animals are candidates to be the source of recombinant proteins at an industrial scale. Although the first recombinant protein produced by transgenic animals is expected to be in the market in 2000, a certain number of technical problems remain to be solved before the various systems are optimized. Although the generation of transgenic farm animals has become recently easier mainly with the technique of animal cloning using transfected somatic cells as nuclear donor, this point remains a limitation as far as cost is concerned. Numerous experiments carried out for the last 15 years have shown that the expression of the transgene is predictable only to a limited extent. This is clearly due to the fact that the expression vectors are not constructed in an appropriate manner. This undoubtedly comes from the fact that all the signals contained in genes have not yet been identified. Gene constructions thus result sometime in poorly functional expression vectors. One possibility consists in using long genomic DNA fragments contained in YAC or BAC vectors. The other relies on the identification of the major important elements required to obtain a satisfactory transgene expression. These elements include essentially gene insulators, chromatin openers, matrix attached regions, enhancers and introns. A certain number of proteins having complex structures (formed by several subunits, being glycosylated, cleaved, carboxylated...) have been obtained at levels sufficient for an industrial exploitation. In other cases, the mammary cellular machinery seems insufficient to promote all the post-translational modifications. The addition of genes coding for enzymes involved in protein maturation has been envisaged and successfully performed in one case. Furin gene expressed specifically in the mammary gland proved to able to cleave native human protein C with good efficiency. In a certain number of cases, the recombinant proteins produced in milk have deleterious effects on the mammary gland function or in the animals themselves. This comes independently from ectopic expression of the transgenes and from the transfer of the recombinant proteins from milk to blood. One possibility to eliminate or reduce these side-effects may be to use systems inducible by an exogenous molecule such as tetracycline allowing the transgene to be expressed only during lactation and strictly in the mammary gland. The purification of recombinant proteins from milk is generally not particularly difficult. This may not be the case, however, when the endogenous proteins such as serum albumin or antibodies are abundantly present in milk. This problem may be still more crucial if proteins are produced in blood. Among the biological contaminants potentially present in the recombinant proteins prepared from transgenic animals, prions are certainly those raising the major concern. The selection of animals chosen to generate transgenics on one hand and the elimination of the potentially contaminated animals, thanks to recently defined quite sensitive tests may reduce the risk to an extremely low level. The available techniques to produce pharmaceutical proteins in milk can be used as well to optimize milk composition of farm animals, to add nutriceuticals in milk and potentially to reduce or even eliminate some mammary infectious diseases.

Introduction

Proteins are the molecules, which have the most numerous biological activities in living organisms. Yet, they are traditionally only marginally used as therapeutic molecules. This is dearly due to the fact that their administration must be done by injection but mainly because most of them are not available or not known.

Genetic engineering offers now the possibility to know virtually all the proteins of a living organism but also to prepare them at an industrial scale as recombinant proteins. After the first success which allowed to prepare human insulin and growth hormone, it appeared that many proteins could not be produced by recombinant bacteria. In some cases, bacteria do not synthesize a foreign protein in large amount. In other cases, the protein is in the form of aggregates, which cannot be recovered easily. On the other hand, many proteins potentially utilizable as pharmaceuticals cannot be synthesized in a satisfactory active form by bacteria. In these cases, the proteins must be obtained from recombinant animal cells. Fermentors containing hybridoma, CHO cells, Sf9 insect cells infected by recombinant baculovirus or other cell lines are extensively used to synthesize antibodies, human erythropoietin, hepatitis B antigen, human factor VIII etc. Although efficient, this approach has intrinsic limitations. The heaviest is probably the fact that, despite quite significant improvement during the last decade, animal cells in culture generally do not produce large amount of recombinant proteins. This is obviously due to the fact that a fermentor contains a relatively limited number of cells, which are maintained in non-optimal metabolic conditions. High capacity fermentors can produce relatively large quantities of recombinant proteins but not at a low cost.

This reality was perceived years ago and after the generation of the first transgenic mice, it was suggested that animals could become living fermentors (Palmiter et al., 1982). The demonstration was given in 1987 when ovine β-lactoglobulin (Simons et al., 1987) and human tissue plasminogen activator (Gordon et al., 1987) were obtained in the milk of transgenic mice. This pioneer work was confirmed since more than 100 foreign proteins have been produced experimentally from different organs and in several animal species. The first protein obtained from transgenic goat milk is expected to be in the market in 2000. Yet, a certain number of problems remain to be solved before the process is optimized.

A certain number of reviews have been written on the subject during the last decade (Houdebine, 1994; Colman, 1996; Rosen et al., 1996; Clark, 1998; Wall, 1999; Rudolph, 1999). The present paper aims at reporting the major successes obtained so far and to analyze the hurdles which remain to generate transgenic animals being more reliable bioreactors.

The different animal systems to produce recombinant proteins

Blood

Serum, which collects secretion from many tissues, may be the source of recombinant proteins. Human α1 antitrypsin synthesized essentially in liver was thus obtained at a high level from the serum of transgenic rabbits. This protein seemed matured in an appropriate manner (Massoud et al., 1991). One limitation in this case was the difficulty to separate the recombinant from the endogenous protein. The replacement of the endogenous gene by the human genes using homologous recombination would solve this problem. Recombinant antibodies were also found in the blood of transgenic pigs and rabbits (Lo et al., 1991; Weidle et al., 1991, Limonta et al., 1995). However, the antibodies were present at a relatively low concentration and they were hybrid molecules containing chains from the endogenous antibodies. Replacement of loci harbouring the antibody genes by human loci may allow mice to synthesize human antibodies (Mendes et al., 1997). The transfer of human chromosome 2 in mouse has been achieved using ES cells. This additional chromosome was transmitted to progeny and was able to generate functional human antibodies after immunisation of the animals (Tomizuka et al., 1997).

Although serum from transgenic animals is an abundant by-product of slaughterhouses it does not appear presently as a potential source for many recombinant proteins. Indeed, many proteins are poorly stable in serum and some of them may hamper the health of the animals.

Human hemoglobin has been obtained in the reticulocytes of transgenic swine (Sharma et al., 1994). This molecule was present at a relatively high level and it was functional. However, a large proportion was formed of hybrid molecules containing also the pig globins. The replacement of pig globin genes by the corresponding human genes would probably solve this problem.

A few experimental data suggest that reticulocytes might be the source of recombinant non-secreted proteins. Efficient and specific promoters driving the expression of foreign genes are available (Sharma et al., 1994). Enzymes or peptides might thus be stored in reticulocytes from transgenic animals and extracted after having been collected from slaughterhouse.

Urine

Urine is an abundant biological fluid already used to prepare proteins such as gonadotropins for pharmaceutical use. A recent work indicated that the human growth hormone gene driven by the promoter of the mouse uroplakin II gene was expressed specifically in urothelium. Up to 100–500 ng/ml of human growth hormone was found in urine. This system may be useful if it happens that the foreign protein is matured in a more appropriate manner in urothelium than in the mammary cell or if the side-effects of the protein are less deleterious for the animals (Kerr et al., 1998).

Seminal plasma

Seminal plasma is a relatively abundant biological fluid in some species and it can be easily collected. This is the case for pig. The promoter of the mouse P12 gene that promotes expression specifically in male accessory sex gland has been used to synthesize the human growth hormone. The hormone was found in the seminal plasma from transgenic mice at a concentration as high as 0.5 mg/ml (Dyck et al., 1999).

This system, as urine, may have specific advantages. In both cases, it is not known how complex proteins are matured and secreted.

Egg white

Egg white, as milk, is an abundant fluid containing huge amount of proteins and being secreted out of the body. It is thus expected to be an excellent system to produce recombinant proteins. Yet, it has not been implemented so far, essentially for technical reasons.

Generating transgenic birds remains a difficult task with limited success. Microinjection of isolated gene into on cell embryo followed by *in vitro* development is one possibility (Naito, 1997). The use of the transposon Mariner considerably enhances the yield of transgenesis (Verrinder-Gibbins, 1998; Scherman et al., 1998). The use of ES cells to generate chimeric chicken is a very attractive approach which met some success (Etches et al., 1997; Kunita et al., 1998; Etches, 1999). The interest of this protocol still remains limited by the fact that the foreign gene is not reproducibly transmitted to progeny.

Retroviral vectors have been extensively studied (Ronfort et al., 1997). When injected in the vicinity of primordial germ cells of developing chicken embryos, the retroviral vectors are integrated into the genome, and the foreign gene are found in progeny with a low but reasonable frequency. These tools recently led to the production of foreign proteins in the egg white of transgenic chicken (Ivarie, 1999).

Silk worm cocoon

The cocoon of insects contains large amount of a few proteins which form silk. From this point of view, this system shares a certain number of properties with milk and egg white. Silk worm is an interesting biological model and it is also a natural producer of silk for industry. This urged several laboratories to generate transgenic silk worms. This was achieved successfully after microinjection of linearized plasmids into eggs (Nagaraju et al., 1996). The use of baculovirus vectors was also shown to be efficient (Yamao et al., 1999). The most promising approach seems presently to rely on the use of a piggy Bac transposon-derived vector (Tamura et al., 1999).

Silk worm can be easily reproduced into large number of individuals. It is therefore, a good candidate to be a living fermentor. The efficiency of foreign gene expression and protein maturation still remains to be evaluated.

Milk

Milk is currently the best available bioreactor (Houdebine, 1994; Colman, 1996; Clark, 1998; Wall, 1999; Rudolph, 1999). Extensive studies have shown that it can be the source of a variety of recombinant proteins, some of them being rather complex molecules.

Among the proteins which can be mentioned are: human IGF1 (Zinovieva et al., 1998), human NGF-β (Coulibaly et al., 1999), hGH (Devinoy et al., 1994), human lysozyme (Lee et al., 1998), human lactoferrin (Platenburg et al., 1994), human erythropoietin (Massoud et al., 1996), human thrombopoietin (Sohn et al., 1999) and human parathyroid hormone (Rokkones et al., 1995). Interestingly, naturally complex proteins have been secreted in milk in a fully functional form. This is the case for human fibrinogen composed

of three subunits (Prunkard et al., 1996). This was also observed for human collagen obtained as a mature molecule after coinjecting the genes coding for the two subunits and for the enzyme which modifies the proteins posttranscriptionnally (John et al., 1999). The human extracellular superoxide dismutase which is formed of two glycosylated homodimers containing one copper ion was also found in milk at the concentration of several milligram per millilitre (Strömqvist et al., 1997). Active recombinant immunoglobulin capable of neutralizing coronavirus was obtained in mouse milk (Castilla et al., 1998). An exotic protein, spider silk, known for its exceptional mechanical properties has been recently produced in mouse milk (Karatzas et al., 1999).

These examples leave no doubt on the capacity of the mammary gland to synthesize, mature and secrete foreign proteins. Apart from these successes, a certain number of failures occurred for various reasons. Some of them are purely technical such as the generation of transgenic mammals. Others, such as protein maturation or secretion, are more fundamental. These points are considered in the following paragraphs.

The generation of transgenic mammals

Microinjection

The direct microinjection of linear DNA fragments into pronuclei defined in 1981 remains the method of choice for the prolific species (mouse, rat, rabbit and pig). An improvement of the method was successful for ruminants. In these species and mainly in cow, the rate of foreign gene integration is very low. On the other hand, embryos generated *in vivo* after superovulation and recipient females are particularly costly. The preparation of one cell embryos after *in vitro* oocyte maturation and fertilization considerably reduced the cost of the experiment. The possibility to develop the microinjected embryos until the blastocyst stage allows the elimination of those which cannot survive. This reduces the number of recipient females. Ideally, the identification of the putative transgenic embryos would still reduce the number of recipient females. The PCR technique which cannot easily make a distinction between the integrated and the free foreign DNA proved to be inappropriate (Page et al., 1995). The best way currently relies on the use of a marker gene coinjected with the gene of interest. Although some of the selected embryos harbour only the marker gene and others are eliminated since they have only the gene of interest, this method is presently the best approach to reduce at most the number of recipient females. The green fluorescent protein (GFP) seems presently the best marker. One major advantage is that this protein can be visualized with a non-invasive test. Alternatively, fluorescent *in situ* hybridization (FISH) performed in one isolated blastomer can reveal the presence of integrated foreign DNA (Echelard, 1997).

This protocol was applied successfully to generate transgenic cows (Krimpenfort et al., 1991; Hyttinen et al., 1994; Eyestone, 1998). It could be used as well for sheep and goat. Indeed, *in vitro* generation of one cell embryos can also be achieved in these species. However, the techniques have not been defined in details and optimized for these two species. On the other hand, the embryos generated *in vitro* usually show a lower rate of survival after transplantation in recipient females. All things being considered, superovulation has been retained for the generation of one cell sheep and goat embryos rather than the *in vitro* protocol.

One of the major limitations of microinjection is the low rate of foreign DNA integration. Works in progress have shown that episomal vectors capable of being maintained as circular genomes can be used to generate transgenic animals with a very high efficiency (Attal et al., 1997; Vos, 1998). Alternatively, linear artificial chromosome might become available in future (Willard, 1998). Significant improvements of these vectors are required before they can become relevant tools to facilitate preparation of recombinant proteins from milk. Episomal vectors capable of being maintained during the life of the animal but not transmitted to progeny might probably be an attractive compromise.

Cell transfection and embryo cloning

The technique defined to generate cloned sheep by transferring nuclei from somatic cells into enucleated oocytes was used successfully to obtain transgenic sheep harbouring the human factor IX gene (Schnieke et al., 1997). This approach proved to be efficient also for goat (Baguisi et al., 1999). It can be extended to all species in which the cloning technique is efficient. This is obviously the case for cow.

This method presently appears as definitely more attractive than microinjection. Indeed, about 2.5 times less animals were required to generate the same number of transgenic sheep by cloning (Schnieke et al., 1997). One of the advantages of the cloning approach

is that the foreign gene is transferred in cultured cells. This allows the selection of cellular clones harbouring a limited number of integrated and intact genes. The cells can be taken from females and thus generate females. This accelerates the production of milk from the founder animals. Cells can be stored frozen and reused to generate additional transgenics.

The overall yield of the method becomes dependent essentially of the embryo cloning efficiency. The cloning of pig embryo which occured recently was expected for several reasons and particularly to prepare organ for xenografting to humans. It is not certain yet that this would compete favorably with the conventional microinjection. The same is true for the other prolific species.

The major advantage of the cloning technique is that it can lead to the replacement of a targeted gene by homologous recombination. Several lambs in which a gene was replaced in cultured somatic cells were generated recently (Ayares, 1999). This theoretically allows the introduction of a foreign gene in the casein locus, in whey protein genes or at any other selected site.

Independently of the method of gene transfer, the cloning technique may contribute quite significantly to rapidly establish a herd of transgenic animals producing pharmaceuticals from the animals producing the highest level of the recombinant proteins.

The generation of chimeric animals from multipotent cells

Gene replacement has currently being performed in mouse for more than 10 years but not in other species. Homologous recombination for gene replacement can be obtained virtually in cell types but only multipotent cell were really useful for this purpose. Indeed, only multipotent cells lines from embryos (ES cells) or from primordial germ cells (EG cells) have the capability to participate to the generation of chimeric animals which are then mosaic for the genetic modification. Reliable multipotent cell lines have been obtained only from a few strains of mice. In other species, the cells loose their multipotency during the culture which is necessary to select the cells in which gene replacement occurred. These cells can then participate only moderately to the development of chimeric embryos and they do not transmit the genetic modification to progeny. All the groups working with rat, rabbit, chicken, pig or ruminants did similar observations. These repeated failures can be attributed to the fact that the biochemical status of multipotent cells has never been defined clearly (Fléchon, 1997; Mc Laren, 2000). Publications suggest each year that slight progress has been done. One of the most recent publications indicates that primordial germ cells from pig were able to generate animals with a high degree of chimerism, suggesting that the multipotent cells added to the embryos might have participated to the formation of germ cells of the chimera (Mueller et al., 1999).

Gene transfer into sperms and oocytes

The introduction of foreign genes before fertilization into germ cells is a logical approach, which did not meet clear success until recently. The incubation of sperm with DNA solution before fertilization gave only a very small proportion of transgenic animals with rearranged genes in most cases. Another protocol originally defined for xenopus was shown to be efficient in mouse. The sperms are first treated by mild detergents to destabilize their membranes. After an incubation in a DNA solution, the sperms have to be microinjected into oocytes to fertilize the oocyte (Perry et al., 1999; Robl, 1999). The efficiency of the method is therefore highly dependent of this of ICSI (intracytoplasmic sperm injection). This method met success in no other mammals than mouse so far.

Microinjection of linear DNA into oocytes never led to the reproducible generation of transgenic animals. The use of retroviral vectors has recently led to a success in cow (Chan et al., 1999) and it is being extended to non-human primates. Retroviral particules containing the envelope from vesicular somatic virus were injected between *zona pellucida* and the membrane of the oocyte at a period when the nuclear membrane is absent. The efficient infection was followed by an easy access to the nucleus and a high rate of foreign gene integration. This method might be more efficient than DNA microinjection. It is not certain that it will be, in time, more attractive than the method implementing the cloning technique.

The expression of the transgenes

Numerous experiments have shown that the level and specificity of expression of a gene construct used as transgene cannot be easily predicted. DNA addition by microinjection generates lines of animals expressing the foreign gene at quite different levels.

It is admitted that this phenomenon is due to a large extent to a position effect (Dobie et al., 1996). Transgenes are poorly or not expressed when integrated in centromeres or telomeres where DNA is inactive and organized in heterochromatin. Experiments carried out *in vitro* using cultured mammary cells and *in vivo* with transgenics revealed that the gene constructs optimized to work in transfected cells may be poorly efficient when transferred into mice (Petitclerc et al., 1995).

These experiments show with no ambiguity that at least two independent points should be taken into consideration to prepare potent expression vectors (i) the intrinsic capacity of the construct to transcribe efficiently the cDNA or the gene (ii) the potency of the same vectors as transgenes. The first parameters can be evaluated with transfected cells *in vitro* whereas the second property of vectors can be estimated only in transgenic animals. This explains why only limited number of experiments have been performed to evaluate the efficiency of expression vectors *in vivo*. For most research projects, transgenic mice synthesizing limited amount of the foreign proteins are sufficient to give a satisfactory answer to experimenters. The same is not true when a high level of expression is needed and this is the case for the production of recombinant proteins.

Another well-known problem with transgenesis is their faculty to be expressed at a low level in various tissues in which the utilized promoter is not expected to work. It is admitted that this ectopic expression is due to a position effect. Indeed, chromatin contains many transcription enhancers, which can stimulate expression of transgene integrated in their vicinity.

These observations suggest that quite different problems must be solved to generate satisfactory expression vectors in a reliable manner.

The optimization of the transcribed region of the transgenes

Each group of living organisms have a preferential use of codons (Fox, 1987). Using synthetic genes may allow the best adaptation of the codons to the mammary cell machinery. The initiation AUG codon is better utilized when it is surrounded by the consensus Kozak sequence GCCA/GCCAUG G. If absent, this sequence may be added by *in vitro* mutation.

In a certain number of cases, it has been observed that proteins naturally synthesized and secreted at a high rate are abundantly produced in milk. This is the case for growth hormone (Devinoy et al., 1994), α1-antitrypsin (Wright et al., 1991) etc. On the contrary, proteins such as human factor VIII which are present at a low concentration in blood are not easily expressed in milk (Niemann et al., 1996; Paleyanda et al., 1997). The same has been observed for proteins not naturally secreted such as intracellular enzymes or viral antigens. In these cases, the proteins might not be efficiently recognized by chaperones in the endoplasmic reticulum and Golgi apparatus or contain signals for a targeting to cell compartments other than the reticulum. For such proteins, little improvements are expected from modifications of the coding region. The minimum modification to be done is to add a signal peptide in the NH2 end of the protein to allow its secretion.

Introns play an essential but complex role in gene expression. They are often not necessary for the expression of a cDNA in transfected cells. Experiments carried out with several genes and promoters have shown that at least one intron, preferably added before the cDNA, is required (Palmiter et al., 1991). The introns have quite different efficiency (Petitclerc et al., 1995). This may be due to two independent mechanisms. The elimination of introns by exon splicing involves multiple signals on mRNA and proteins binding to specific sequences (Horowitz & Krainer, 1994). The choice of an intron must take this parameter into account. Introns and specially the first intron of genes contain binding sites for transcription factors. This contributes to maintain chromatin in an open and active form around the cap. These introns must be used preferably. Alternatively, sequences for the binding of transcription factors may be added within the intron (Petitclerc et al., 1995).

One of the functions of introns is to favor the translocation of mRNA from the nucleus to the cytoplasm. Sequences playing this role have been found in several genes devoid of introns (Huang & Carmichael, 1997). The real efficiency of these sequences added to transgenes has not been described so far.

A certain number of cDNAs contain cryptic splicing sites which recombine with the splicing donor site of the introns located in the upstream. This was observed with the human factor IX cDNA (Clark, 1998). Mutation in the cDNA may eliminate the splicing site. It is not known if the above mentioned sequences might be an alternative to mutation of the splicing site.

The 5′UTRs (untranslated region) of the mRNA allow a better translation efficiency when they are

poorly structured. Sequences favoring translation such as IRES (internal ribosome entry site) may be added before the initiation codon (Houdebine & Attal, 1999). The 3′UTRs sometimes contain sequences contributing to stabilization of mRNA. Available informations on this point are too rare to be presently useful.

Transcription terminators have different potency. Those from growth hormone or β-globin genes are reputed to give satisfactory results.

One possible strategy may consist in introducing the foreign cDNA within a milk protein gene. Minigenes containing the first and the last introns have also been used. This proved to be a good approach in some cases but not in others. It cannot therefore, be retained as the best solution.

In a certain number of cases, several cistrons must be expressed simultaneously. Bicistronic constructs may in principle give satisfactory results. This implies that IRESs are added between both cistrons. In practice, it appears that IRESs have quite variable potency and that they must be added at about 100 nucleotides after the termination codon of the first cistron to be fully efficient. Moreover, bicistronic mRNA may not be translated at a high rate (Houdebine & Attal, 1999). It seems therefore, preferable to co-inject the constructs to be expressed simultaneously or to link them within the same plasmid if high level of expression are wanted.

The factors controlling transcription of transgenes

The promoters

To express foreign genes into the mammary gland, milk protein gene promoters must be used. The known genes have described by Mercier and Vilotte (1997). These promoters showed different potency. Those of K and αS2-casein are particularly weak. All the other promoters are being used with variable success. The MMTV LTR is also a candidate. Its potency and its specificity may be insufficient for this purpose.

Among the different milk protein genes tested and containing only a few kilobytes of DNA as promoters, only two of them ovine β-lactoglobulin and rat whey acidic protein (WAP), were expressed in a satisfactory manner as transgenes. Their expression occurred in all mice as a function of the copy number with no ectopic transcription (Withelaw et al., 1992; Dale et al., 1992). In both cases the promoters were of variable and unpredictable efficiency when associated with foreign genes. This suggests that signals of unknown nature and located within the genes contribute to the expression of the transgenes. Unexpectedly, these signals loose a large part of their efficiency when foreign cDNAs are introduced within the genes.

The real capacity of the different milk protein gene promoters cannot be easily evaluated. A comparison can be done. The human GH gene has been used as a reporter by several groups (Günzburg et al., 1991; Reddy et al., 1991; Tojo et al., 1993; Devinoy et al., 1994; Ninomiya et al., 1994; Wen et al., 1995; Hirabayashi et al., 1996; Cerdan et al., 1998; Oh et al., 1999). From these studies, it appears that the rabbit WAP promoter (6.3 kb) is more efficient than the mouse WAP promoter (2.6 kb). The variations of the expression level in the individual mice indicate that none of these promoters can, alone, give expression of transgenes in a reproducible manner.

A co-injection of a gene construct with the whole β-lactoglobulin known to be highly expressed was shown to rescue the expression of the foreign co-integrated gene. This effect was dependent on the transcription of the β-lactoglobulin gene but not of its translation (Yull et al., 1997). Although helpful, the addition of β-lactoglobulin gene appears insufficient to bring expression of associated genes to a satisfactory level in all cases.

The distal regulatory elements

Experiments carried out with different genes have indicated that long genomic DNA fragments (100–300 kb) are highly efficient to express the gene they contain in transgenic mice. This proved to be the case for human (Fujiwara et al., 1999a) and goat (Stinnakre et al., 1999) α-lactalbumin contained in YAC and BAC vectors, respectively. In both cases, the genes were expressed in all transgenic mices in a copy dependent manner.

Interestingly, the DNA fragment containing the human α-lactalbumin was highly efficient to express hGH gene (Fujiwara et al., 1999b).

Some of the regulatory elements located far upstream or downstream of the genes have initially been named insulators. Indeed, it is generally believed that a transgene is unduly activated or extinguished by the action of host genomic regulators located in its vicinity. A certain number of experiments indicate that the situation is far more complex.

The insulating effect was first attributed to MAR (matrix attached region) sequences present in the DNA fragments surrounding the genes. These fragments were shown to enhance associated transgene expression. MARs are generally AT rich regions, which

bind to the nuclear matrix where many transcription factors are concentrated. It is admitted that genes are bound to the nuclear matrix when they are actively transcribed. Additional studies have demonstrated that the insulating effect was not solely due to MARs but to other unidentified sequences located in their vicinity (Sippel et al., 1997; Bonifer, 1999). MARs seem still to contribute the expression of associated genes and transgenes by modifying DNA structure through DNA topoisomerase action (Adachi et al., 1989) and inhibition of methylation (Forrester et al., 1999).

Gene insulators are DNA sequence, which prevent action of the regulatory elements of a gene on a neighbour gene. Such sequences have been identified in Drosophila genome (Dorsett, 1999). Experimentally, insulators are defined as elements preventing the activation of a promoter by a neighbour enhancer. An insulator was found in the 5′HS4 region of the LCR (locus control region) from the chicken β-globin locus (Recillias-Targa et al., 1999). Insulators are not considered as capable *per se* of stimulating gene or transgene expression.

The effect of insulators on transgene expression is generally considered as being essentially a protective effect against the action of genomic extinguisher. A certain number experiments do not support this view. Transgenic rabbits harbouring the human DAF and CD59 under the control of the human EF1α gene do not express the foreign genes at a high rate in the six lines examined (Taboit-Dameron et al., 1999). It is unlikely that in all cases the transgenes were integrated in the vicinity of genomic extinguishers. The addition of the 5′HS4 from chicken β-globin locus to the vectors was sufficient to allow a high expression of the foreign genes in all the lines of transgenic rabbits (Taboit-Dameron et al., 1999). A similar effect was observed with the transerythritin gene promoter in liver (Wang et al., 1996) and the bovine αS1-casein gene promoter in the mammary gland (Echelard, 1998). This suggests that the 5′HS4 region contains not only an insulator but also elements capable of maintaining chromatin in an active form. It is more and more believed that these elements are chromatin openers which locally induce an hyperacetylation of histones and a demethylation of DNA (Pikaart et al., 1998; Bonifer, 1999). It becomes, therefore, more and more difficult to make a clear distinction between chromatin openers and classical enhancers. They both seem to act by increasing the frequency of chromatin opening rather than by interacting directly with the complex of transcription initiation. Variegation in expression has often been observed with transgenes. Chromatin openers found in LCR and enhancers seem essentially to reduce the intensity of variegation (Walters et al., 1996). Alternatively, the idea that insulators have the capacity not only of preventing the activation by neighour enhancers but also of blocking the formation of inactive chromatin is attractive (Bell & Felsenfeld, 1999).

The question remains to know why transgenes are so frequently silent. Some general rules are progressively emerging. A transgene seems to have less chance to be expressed efficiently if it contains non animal sequences, if it is devoid of intron and if it is integrated in multicopy (Dorer, 1997; Garrick et al., 1998). An interesting hypothesis is that the cellular mechanisms which inactivate retroviral and transposon sequences by DNA methylation and deacetylation of histones are also those which extinguish transgenes (Fire, 1999). The effect of the chromatin openers and distal enhancers might be to inhibit the mechanism of transgene silencing (Santoso et al., 2000). To be efficiently expressed, a transgene should therefore contain introns, to be devoid of plasmid or synthetic sequences, not to be integrated in multicopies or to be associated with an appropriate LCR. This situation is encountered with the long genomic DNA fragments.

Another mechanism has not been described in detail. Ectopic expression of transgenes is thought to be due to the action of neighbour genomic enhancers. The addition of appropriate insulators to the transgenes should reduce or even suppress this effect. Alternatively, the ectopic expression may result from a cryptic basal transcription at the site of integration. Such basal transcription has been observed in different genomic regions (Travers, 1999) namely in the human β-globin locus (Ashe et al., 1997).

Independently, cryptic transcription of both DNA strands might generate double strand DNA which induces RNA interference which leads to a specific degradation of the mRNA coded by the transgene and to its apparent silencing (Fire, 1999; Bosher & Labouesse, 2000).

The possible vectors for a special control of transgene expression

The gene constructs may be introduced within a milk protein gene by conventional homologous recombination. The use of restriction sites, which cleave both DNA strands, may improve the efficiency of homologous recombination (Cohen-Tannoudji & Babinet, 1998). A simplified protocol may rely on the use of the Cre-loxP system. This system is generally implemen-

ted to eliminate a given integrated sequence flanked by two loxP sequences. The recombination of the loxP sequences is triggered by the presence of the Cre recombinase (Wagner et al., 1997). A loxP sequence preintegrated in a milk protein gene may be the targeted site of integration for a gene construct flanked by two loxP motives (Rucker & Piedrahita, 1997; Kolb et al., 1999).

The advantage of these protocols is that relatively simple and standard gene constructs are expected to be sufficient since they are integrated in genomic locus containing the required regulatory elements. The integration of a foreign gene in a milk protein gene implies the inactivation of the latter. It has been shown that the inactivation of β-casein gene does not alter milk secretion. On the contrary, milk devoid of αs1-casein is no more secreted with high efficiency (Chanat et al., 1999).

Several systems allowing the control of transgene expression by exogenous inducers not acting on endogenous genes are available. The most popular relies on the use of tetracycline and analogues. This system allowed the conditional expression of goat α-lactalbumin gene in transgenic mice (Soulier et al., 1999). In the best conditions, the transgenic may in this way be expressed only in the mammary gland during lactation. The promoter which governs the expression of the gene of interest is potent and it drives the synthesis of the corresponding protein at a high level. In practice, a background expression due to an imperfect control of the gene coding for the tetracycline-sensitive transcription factor and to ectopic expression out of the mammary gland may reduce the advantage of this system. A selection of the mouse lines expressing the activator in an appropriate manner must be previously done. Improved versions of this tool have been recently proposed (Blau & Rossi, 1999; Forster et al., 1999). It implies the simultaneous use of an activator and a repressor both sensitives to tetracycline or doxycycline. This system may eliminate the background but not the ectopic expression.

The post translational events

The maturation of the proteins

After their biosynthesis, many proteins are subjected to biochemical modifications including specific cleavage, folding, subunit assembly, glycosylation, carboxylation, amidation, etc. These mechanisms are dependent on cellular enzymes, which are present at variable concentration in the different cell types.

Glycosylation is undoubtedly one of the most important post-translational event for therapeutic proteins (Fussenegger et al., 1999). Glycosylation may be required for the biological activity of the proteins. This is the case for gonadotropins and to some extent for antibodies (Wright & Morrison, 1997). Glycosylation is essential for the stability of many proteins in blood circulation.

The mammary cell naturally secretes proteins which are N-or O-glycosylated. Recombinant proteins found in milk are glycosylated but not always in an appropriate manner. Human antithrombin III was shown to be not stoichiometrically sialylated. Yet, this protein extracted from the milk of transgenic goats is expected to be on the market in 2000. Indeed, the lack of sialylation reduces the stability of the molecule *in vivo* but it does not hamper its biological activity (Meade, 1999). Other proteins such as α1-antitrypsin (Clark, 1998) or EC superoxide dismutase (Strömqvist et al., 1997) were glycosylated in a manner almost similar to this of the native proteins. Unexpectedly, the bile salt-stimulated lipase was defective of O-glycosylation (Strömqvist et al., 1996). This fact has not been explained. Indeed, this protein is naturally secreted in milk in the O-glycosylated form. The reasons why some recombinant proteins are not correctly glycosylated are particularly complex. The glycosylating enzymes may be limiting, specially when very high amounts of the foreign proteins are synthesized. The addition of the genes coding for the glycosylating enzymes to the animals by transgenesis is conceivable. This approach met some success with some cultured cell lines. A moderate expression of the genes coding for glycosidases is probably needed to avoid disturbance of the cellular machinery. The poor glycosylation of some proteins may result from their inappropriate folding in the endoplasmic reticulum and the Golgi apparatus not providing the enzymes with a free access to the glycosylation sites.

The human protein C produced in the milk of mice was poorly active. A study of the molecule revealed that it was not quantitatively cleaved. Subunits generation and assembly could thus not occur. The action of the furin transgene allowed the native proteins C to be processed and active (Drews et al., 1995). As opposed, the human protein C from transgenic pig was fully active. The human factor IX was carboxylated by the mammary cell (Clark, 1998).

The stability of transgene expression

In mouse, the high expression of a transgene reduced somewhat the synthesis of endogenous milk proteins (Mc Clenaghan et al., 1995). This fact is not really surprising. Indeed, during lactation in the rabbit, up to 20% of the milk protein mRNAs remain free and not associated to form polysomes (Houdebine unpublished data). This suggests that the translation machinery is saturated. Exogenous mRNAs are then expected to compete with the milk protein mRNAs. The same phenomenon has not been observed in ruminants (Colman, 1996).

The expression of a transgene was essentially at a constant level in the different individuals of a line over number of generations (Colman, 1996). The co-suppression of transgene expression observed repeatedly in plants is therefore, at most marginal in animals. An exception was reported: the bovine α-lactalbumin transgene in mouse was expressed at a highly variable degree in the individuals of a given line (Bleck & Bremel, 1994). Another important point is the stability of the transgene itself in the long term. Experiments carried out in mouse indicated that transgeneses are generally quite stably integrated in host genome (Aigner et al., 1999).

The in vitro *and* in vivo *systems to predict the efficiency of a gene construct*

A few mammary cell lines are available and extensively used in different laboratories. The most popular is the HC11 mouse line. The cells can at best predict the intrinsic potency of a construct for transcription but not the level of expression in transgenic animals. The cell lines are not expected to be able to reflect all the events, which mature the proteins post-transcriptionally.

The direct introduction of a gene construct into the mammary gland of a lactating animal has been performed with a retroviral vector (Archer et al., 1994) or with a gene gun (Kerr et al., 1996). It can at best provide relevant informations on the post translational modifications of the recombinant proteins but not predict the transcription efficiency of the construct.

The side-effects of the recombinant proteins on the animals

The recombinant proteins produced in milk are generally to be used in humans. Their chance to be active in the animals is high. Side-effects on the animals were observed with human erythropoietin (Massoud et al., 1996) with hGH (Devinoy et al., 1994) with bGH (Thépot et al., 1995) with *zona pellucida* glycoprotein mZP3 (Litscher et al., 1999) and probably with some others. In several cases (Devinoy et al., 1994; Thépot et al., 1995; Bishoff et al., 1992), the concentration of the recombinant proteins was much higher in blood during lactation. In one mouse line at least, the hGH was found in blood only during lactation. The idea that the lactogenic hormones enhanced the ectopic expression of the transgenes is unlikely. Rather, a small proportion of the recombinant proteins escapes from the mammary gland and migrates to blood. This may result from a leakiness of the mammary epithelium or to a non-strictly vectorial secretion at the apical side of the cells. It is interesting to note that whey proteins, namely WAP, are normally found in blood of lactating animals (Grabowski et al., 1991). Caseins organized in large micelles are not present in blood during lactation. Only whey and recombinant proteins in solution in the lactoserum can cross to some extent the mammary epithelial barrier. This suggests that a protein having a deleterious side-effect in the animal may not be produced without any problem in milk even if the transgene is strictly controlled by the tetracycline-dependent system. More specific effects were observed at the mammary gland level. WAP impaired mouse and pig mammary gland development (Shamay et al., 1992). Human EC superoxide dismutase gene expressed in rabbit mammary gland reduced quite significantly milk production (Houdebine, 1998).

The purification of recombinant proteins

The purification of the recombinant proteins from milk raises generally no particular problem. Milk contains little amount of proteases. Casein can be removed easily by non-drastic procedures. Chromatography may, on a case by case basis, lead to a high purity of the proteins (Wright & Colman, 1997; Van Cott et al., 1996). However, it should be kept in mind that milk is a relatively complex biological fluid and that the complete elimination of some of its components may not be easy in some cases.

Particular difficulty may be encountered when the recombinant protein is similar to an abundant milk protein. This is the case of human serum albumin which cannot be separated easily from the same protein from the animals.

Specific breeding conditions and selection of animals among herd non-contaminated by prions are considered as sufficient to prepare safe products.

Conclusion and perspective

The techniques leading to the production of recombinant proteins in milk have now reached a certain degree of maturity. The fact that human antithrombin III from goat milk is expected on the market in 2000 illustrates this point.

These techniques may and must be improved. The cloning of embryos with transfected cells as nuclear donors has greatly facilitated gene transfer into ruminants. Yet, the availability of reliable episomal vectors would still simplify the generation of transgenic animals in all species.

Expression vectors have still great progress to make. Gene constructs still often lead to unexpected results in transgenics (Ilan et al., 1996a,b; Barash et al., 1999). The use of episomal vectors independent of host chromatin would probably alleviate some of the expression problems.

The present trends consist in using long genomic DNA fragments as vectors. This is the case for α-lactalbumin gene (Fujiwara et al., 1999; Stinnakre, 1999). Works in progress suggest that regions from the casein cluster could have LCR properties and allow a high and reliable expression of foreign genes (Rijnkels et al., 1999). The same reasoning may be valuable for β-lactoglobulin and WAP genes.

In future, the essential elements controlling the expression of milk protein genes might be combined in an optimum manner to generate compact and highly efficient promoters. Such an approach has recently led to the definition of a regulatory region quite specific of muscle cells and more potent than the natural promoters (Li et al. 1999; Somia et al., 1999).

The mammary gland is presently the only really available animal bioreactor. It has a certain number of competitors which have been depicted above. Transgenic plants are also potentially other potent systems to produce recombinant proteins (Fisher et al., 1999). However, the purification of foreign proteins from plants is not always easy and some of the post-translation modifications of animal proteins are not correctly achieved in plant cells.

Cells in culture may become more competitive systems in the future. It is impressive that functional humanized IgA synthesized by using four independent genes can be secreted from recombinant CHO cells at a concentration as high as 20 μg/ml of medium per 1×10^6 cells for a period of 24 h (Berdoz et al., 1999).

Other competitor systems may emerge in future. Recent works have shown that peptides not directly related to human erythropoietin (EPO) can mimic the action to the hormone (Wrighton et al., 1996). Non peptide analogues may also mimic EPO action (Qureschi et al., 1999). This approach is very attractive since the synthetic molecules may be potentially administered by the oral route. On the contrary, most of the proteins have to be injected repeatedly. Progresses are being made to protect proteins from degradation in the digestive tract and to favor their absorption by the gut. The production of recombinant proteins by bioreactors at a low cost might be a decisive advantage in that case.

The direct transfer of a gene to a living organism may lead to a significant secretion and action of the corresponding protein. Pig growth was in this way accelerated by the transfer of the GHRH gene (Draghia-Akli et al., 1999). This procedure has many advantages over protein injection. These sophisticated systems have little chance to be implemented for many proteins. The production of recombinant proteins thus will most likely remain an important industrial activity for several decades. Several hundreds of proteins for many different purposes are expected to be prepared by bioreactors at a competitive price (Wall, 1999, Harris, 1999). A few recombinant proteins synthesized by bioreactors are currently subjected to clinical tests.

Antibodies seem to be the kind of proteins which will be the most frequently used. Human monoclonal antibodies may, for example, be a good alternative to antibiotics for some infection diseases. Bacteria and yeast can produce simplified antibodies (ScFv and Fab) but less easily Fab′2 or complete molecules. Milk (and possibly other bioreactors) proved to reach this goal in several cases.

Certain categories of proteins have not been produced yet or marginally in milk. This is the case for peptides having antimicrobial activities. These molecules may be an attractive alternative to antibiotics (Yarus et al., 1996; Latham, 1999).

Recombinant membrane proteins can be obtained from milk fat globules. This was the case for CFTR (Di Tullio et al., 1992). The amount of the protein was very low. Yet, milk may potentially be the way to obtain membrane receptors in sufficient amount to define their structure after crystallization. This ap-

proach may be essential to define synthetic molecules acting on the receptors. It is clear that milk may be the source of proteins not only for direct therapeutical use but also for the study of proteins proper. Small animals, namely rabbit, may be the bioreactors of choice for this purpose. Mice may in some cases provide experimenters with enough protein. Indeed, the incubation of the mammary gland on ice may release more than one milliliter of milk in a few hours (Stinnakre et al., 1992). The advantages and drawbacks of the different species have been amply discussed in the previous reviews mentioned above.

The techniques defined for the production of pharmaceuticals in milk may be used as well or to add nutriceuticals in milk or to optimize milk composition (Zuelke, 1998). Human lysozyme and lactoferrin, which have antibacterial activity, are present in cow milk. The concentration of glycoconjugates in milk may also be increased by the expression of the human glycosyl transferase gene (Prieto et al., 1995).

Several systems are being implemented to reduce lactose concentration in milk (Alton et al., 1998; Whitelaw, 1999). The data obtained so far indicate that the absence of lactose prevents milk secretion. Hence, at best only a reduction of lactose concentration is compatible with a normal lactation. IgA directed against viruses infecting the digestive tract may be produced in milk (Saif & Wheeler, 1998; Castilla et al. 1998, Sola et al., 1998). The concentration of IgA receptor in mammary cell may be enhanced. This may potentially favor the accumulation of the protective antibodies in milk (De Groot et al., 1999). Experiments in progress suggest that viral antigens active by the oral route might potentially vaccinate human and farm animals against infection diseases. The nutritional value of farm animal milk may also been improved. The secretion of bovine α-lactalbumin in pig milk increased piglet growth (Bleck et al., 1998; Wheeler, 1999).

Human α-lactalbumin devoid of phenylalamine is present in the milk of transgenic cows. This problem may be the source of amino acids for people suffering from penylkenoneurea. All these data indicate that the bioreactors are now ready, although still imperfect, to enter the industrial world. Recent tools capable of identifying the presence of the modified form of PrP involved in prion disease should allow the early detection of contaminated animals and thus eliminate this biosafety problem.

Note added in proof

Human α-1 antitrypsin was produced in sheep milk after the introduction of the cDNA under β-lactoglobulin gave promoter by homologous recombination in fetal fibroblasts subsequently used to generate transgenic annuals by cloning (McCreath et al. (2000) *Nature* **405** 1066–1069).

References

Adachi Y, Käs E and Laemmli UK (1989) Preferential, cooperative binding of DNA topoisomerase II to scaffold-associated regions. *EMBO J* **8**: 3997–4006.

Aigner B, Fleischmann M, Muller M and Brem G (1999) Stable long term germ-line transmission of transgene integration sites in mice. *Transgenic Res* **8**: 1–8.

Alton E, Griesenbach U and Geddes DM (1998) Milking gene therapy. *Nature Sci* **4**: 1121–1122.

Archer JS, Kennan WS, Gould MN and Bremel RD (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.

Ashe HJ, Monks J, Wijgerde M, Fraser P, Proudfoot NJ (1997) Intergenic transcription and transinduction of the human β-globin locus. *Genes Dev* **11**: 2494–2509.

Attal J, Stinnakre MG, Théron MC, Terqui M and Houdebine LM (1997) The use of episomal vectors for transgenesis. In: Houdebine LM (ed.) *Transgenic Animals: Generation and Use*. (pp. 251–255). Harwood Academic Publishers, Amsterdam.

Ayares D (1999) Gene targeting in livestock. *Transgenic Research Conference*, Tahoe City Aout 1999, 20.

Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, et al., (1999) Production of goats by somatic cell nuclear transfer. *Nature Biotech* **17**: 456–461.

Barash I, Faerman A, Richenstein M, Kari R, Damary G, Shani M and Bissell MJ (1999) *In vivo* and *in vitro* expression of human serum albumin genomic sequences in mammary epithelial cells with β-lactoglobulin and whey acidic protein promoters. *Mol Repro Dev* **52**: 241–252.

Bell AC and Felsenfeld G (1999) Stopped at the border: boundaries and insulators. *Cur Opin Gene Dev* **9**: 191–198.

Berdoz J, Tallichet Blanc C, Reinhardt M and Kraehenbuhl JP (1999) *In vitro* comparison of the antigen-binding and stability properties of the various molecular forms of IgA antibodies assembled and produced in CHO cells. *Proc Natl Acad Sci USA* **96**: 3029–3034.

Bishoff R, Degryse E, Perraud F, Dalemans W, Ali-Hadji D, Thépot D, et al., (1992) A 17.6 kbp region located upstream of the rabbit *WAP* gene directs high level expression of a functional human protein variant in transgenic mouse milk. *FEBS Lett* **305**: 265–268.

Blau HM and Rossi F (1999) Tet B or not tet B: advances in tetracycline-inducible gene expression. *Proc Natl Acad Sci USA* **96**: 797–799.

Bleck GT and Bremel RD (1994) Variation in expression of a bovine alpha-lactalbumin transgene in milk of transgenic mice. *J Dairy Sci* **77**: 1897–1904.

Bleck GT, White BR, Miller DJ and Wheeler MB (1998) Production of bovine α-lactalbumin in the milk of transgenic pigs. *J Anim Sci* **76**: 3072–3078.

Bosher JM and Labouesse M (2000) RNA interference: genetic wand and genetic watchdog. *Nature Cell Biol* **2**: E31–E36.

Bonifer C (1999) Long-distance chromatin mechanisms controlling tissue-specific gene locus activation. *Gene* **238**: 277–289.

Castilla J, Pintado B, Sola I, Sanchez-Morgado JM, Enjuanes L (1998) Engineering passive immunity in transgenic mice secreting virus-neutralizing antibodies in milk. *Nature Biotech* **16**: 349–354.

Cerdan MG, Young JI, Zino E, Falzone TL, Otero V, Torres HN and Rubinstein M (1998) Accurate spatial and temporal transgene expression driven by a 3.8-kilobase promoter of the bovine β-casein gene in the lactating mouse mammary gland. *Mol Reprod Dev* **49**: 236–245.

Chan AWS, Homan EJ, Ballou LU, Burns JC and Bremel RD (1998) Transgenic cattle produce reverse-transcribed gene transfer in oocytes. *Proc Natl Acad Sci USA* **95**: 14028–14033.

Chanat E, Martin P and Ollivier-Bousquet M (1999) αS1-casein is required for the efficient transport of β- and κ-casein from the endoplasmic reticulum to the Golgi apparatus of mammary epithelial cells. *J Cell Sci.* **112**: 3399–3412.

Clark AJ (1998) The mammary gland as a bioreactor: expression, processing and production of recombinant proteins. *J Mamm Gland Biol Neop* **3**: 337–349.

Cohen-Tannoudji M and Babinet C (1998) Beyond 'knock-out' mice: new perspectives for the programmed modification of the mammalian genome. *Mol Hum Reprod* **4**: 929–938.

Colman A (1996) Production of proteins in the milk of transgenic livestock: problems, solutions, and successes. *Am J Clin Nutr* **63**: 639S–645S.

Coulibaly S, Besenfelder U, Fleischmann M, Zinovieva N, Grossmann A, Wozny M, et al., (1999) Human nerve growth factor beta (hNGF-β): mammary gland specific expression and production in transgenic rabbits. *FEBS Lett* **444**: 111–116.

Dale TC, Krnacik MJ, Schmidhauser C, Yang CLQ, Bissell MJ and Rosen JM (1992) High level expression of the rat whey acidic protein gene is mediated by elements in the promoter and 3′ untranslated region. *Mol Cell Biol* **12**: 905–914.

De Groot N, Van Kuik-Romeijn P, Lee SH and De Boer H (1999) Over-expression of the murine polymeric immunoglobulin receptor gene in the mammary gland of transgenic mice. *Transgenic Res* **8**: 125–135.

Devinoy E, Thépot D, Stinnakre MG, Fontaine ML, Grabowski H, Puissant C, et al., (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.

Di Tullio P, Cheng SH, Marshall J, Gregory RJ, Ebert KM, Maede HM and Smith AE (1992) Production of cystic fibrosis transmenbrane conductance regulator in the milk of transgenic mice. *Bio/Tech* **10**: 74–77.

Dobie KW, Lee M, Fantes JA, Graham E, Clark AJ, Springbett A, et al., (1996) Variegated transgene expression in mouse mammary gland is determined by the transgene integration locus. *Proc Natl Acad Sci USA* **93**: 6659–6664.

Dorsett D (1999) Distant liaisons: long-range enhancer-promoter interactions in *Drosophila*. *Curr Opin Gene Dev* **9**: 505–514.

Dorer DR (1997) Do transgene arrays form heterochromatin in vertebrates? *Transgenic Res* **6**: 3–10.

Draghia-Akli R, Fiorotto ML, Hill LA, Malone PB, Deaver DR and Schwartz RJ (1999) Myogenic expression of an injectable protease-resistant growth hormone-releasing hormone augments long-term growth in pigs. *Nature Biotech* **17**: 1179–1183.

Drews R, Paleyanda RK, Lee TK, Chang RR, Rehemtulla A, Kaufman RJ, et al., (1995) Proteolytic maturation of protein C upon engineering the mouse mammary gland to express furin. *Proc Natl Acad Sci USA* **92**: 10462–10466.

Dyck MK, Gagné D, Ouellet M, Sénéchal J, Bélanger E, Lacroix D, et al., (1999) Seminal vesicle production and secretion of growth hormone into seminal fluid. *Nature Biotech* **17**: 1087–1090.

Echelard Y (1997) Genetic mosaicism in the generation of transgenic mice. In: Houdebine LM (ed.) *Transgenic Animals: Generation and Use*. (pp. 233–235). Harwood Academic Publishers, Amsterdam.

Echelard Y (1998) Increasing efficiency of transgenesis. Genetically engineering and cloning animals: Deer Valley Utah USA.

Etches RJ, Clark ME, Verrinder Gibbins AM and Cochran MB (1997) Production of chimeric chickens as intermediates for gene transfer. *Transgenic Animals: Generation and Use*, Harwood Academic Publishers, Amsterdam, pp. 75–82.

Etches R (1999) Avian embryonic stem cells and their application in the pharmaceutical and poultry industries. *Transgenic Animal Research Conference Tahoe City USA* 13.

Eyestone WH (1998) Production and breeding of transgenic cattle using *in vitro* embryo production technology. *Theriogeriology* **51**: 509–517.

Fischer R, Schumann D, Zimmermann S, Drossard J, Sack M and Schillberg S (1999) Expression and characterization of bispecific single-chain Fv fragments produced in transgenic plants. *Eur J Biochem* **262**: 810–816.

Fire A (1999) RNA-triggered gene silencing. *Trends Genet* **15**: 358–363.

Fléchon JE (1997) What are ES cells? In: Houdebine LM (ed.) *Transgenic Animals: Generation and Use* (pp. 157–166). Harwood Academic Publishers, Amsterdam.

Forrester WC, Fernandez LA, Grosschedl R (1999) Nuclear matrix attachment regions antagonize methylation-dependent repression of long-range enhancer promoter interactions. *Gene Dev* **13**: 3003–3014.

Forster K, Helbl V, Lederer T, Urlinger S, Wittenburg N, Hillen W (1999) Tetracycline-inducible expression systems with reduced basal activity in mammalian cells. *Nucleic Acids Res* **27**: 708–710.

Fox TD (1987) Natural variation in the genetic code. *Ann Rev Genet* **21**: 67–91.

Fujiwara Y, Takahashi R, Miwa M, Kameda M, Kodaira K, Hirabayashi M, et al., (1999a) Analysis of control elements for position independent expression of human α-lactalbumin YAC. *Mol Reprod Dev* **54**: 17–23.

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

Fussenegger M, Bailey JE, Hauser H and Mueller PP (1999) Genetic optimization of recombinant glycoprotein production by mammalian cells. *Trends Biotech* **17**: 35–42.

Garrick D, Fiering S, Martin DIK and Whitelaw E (1998) Repeat-induced gene silencing in mammals. *Nature Gen* **18**: 56–59.

Gordon K, Lee E, Vitale JA, Smith AE, Westphal H and Hennighausen L (1987) Production of human tissue plasminogen activator in transgenic mouse milk. *Bio/Technol* **5**: 1183–1187.

Grabowski H, Le Bars D, Chene N, Attal J, Malienou-Ngassa R, Puissant C and Houdebine LM (1991) Rabbit whey acidic protein concentration in milk, serum, mammary gland extract, and culture medium. *J Dairy Sci* **74**: 4143–4150.

Günzburg WH, Salmons B, Zimmermann B, Müller M, Erfle V and Brem G (1991) A mammary specific promoter directs expression of growth hormone not only to the mammary gland, but also to Bergman Glia cells in transgenic mice. *Mol Endocri* **5**: 123–133.

Harris B (1999) Exploiting antibody-based technologies to manage environmental pollution. *Trends Biotech* **17**: 290–296.

Hirabayashi M, Kodaira K, Takahashi R, Sagara J, Suzuki T and Ueda M (1996) Transgene expression in mammary glands of newborn rats. *Mol Reprod Dev* **43**: 145–149.

Horowitz DS and Krainer AR (1994) Mechanisms for selecting 5′ splice sites in mammalian pre-mRNA splicing. *Trends Genet* **10**: 100–106.

Houdebine LM (1994) Production of pharmaceutical proteins from transgenic animals. *J Biotech* **34**: 269–287.

Houdebine LM (1998) The preparation of recombinant superoxide dismutases from the milk of transgenic animals. Mel Paris (ed.) *Superoxide Dismutase: Recent advances and clinical applications* (pp. 239–242).

Houdebine LM and Attal J (1999) Internal ribosome entry sites (IRESs): reality and use. *Transgenic Res* **8**: 157–177.

Huang Y and Carmichael GG (1997) The mouse histone H2a gene contains a small element that facilitates cytoplasmic accumulation of intronless gene transcripts and of unspliced HIV-1-related mRNAs. *Proc Natl Acad Sci USA* **94**: 10104–10109.

Hyttinen JM, Peura T, Tolvanen M, Aalto J, Alhonen L, Sinervirta R, et al., (1994) Generation of transgenic dairy cattle from transgene analyzed and sexed embryos produced *in vitro*. *Bio/Technol* **12**: 606–608.

Ilan N, Barash I, Faerman A and Shani M (1996a) Dual regulation of β-lactoglobulin/human serum albumin gene expression by the extracellular matrix in mammary cells from transgenic mice. *Exp Cell Res* **224**: 28–38.

Ilan N, Barash I, Raikhinstein M, Faerman A and Shani M (1996b) β-lactoglobulin/human serum albumin fusion genes do not respond accurately to signals from the extracellular matrix in mammary epithelial cells from transgenic mice. *Exp Cell Res* **228**: 146–159.

Ivarie R (1999) Validating the Hen as a bioreactor for the production of exogenous proteins in egg whites. *Transgenic Animal Research Conference Tahoe City USA*, 22.

John DCA, Watson R, Kind AJ, Scott AR, Kadler KE and Bulleid NJ (1999) Expression of an engineered form of recombinant procollagen in mouse milk. *Nature Biotech* **17**: 385–389.

Karatzas C, Zhou JF, Huang Y, Duguay F, Chretien N, Bhatia B, et al., (1999) Production of recombinant spider silk (biosteel^{TM}) in the milk of transgenic animals. *Transgenic Animal Research Conference Tahoe City USA*, 34.

Kerr DE, Furth PA, Powell AM, Wall RJ (1996) Expression of gene-gun injected plasmid DNA in the ovine mammary gland and in lymph nodes draining the injection site. *Animal Biotech* **7**: 33–45.

Kerr DE, Liang F, Bondioli KR, Zhao H, Kreibich G, Wall RJ and Sun T (1998) The bladder as a bioreactor: urothelium production and secretion of growth hormone into urine. *Nature Biotech* **16**: 75–79.

Kolb AF, Ansell R, McWhir J and Siddell SG (1999) Insertion of a foreign gene into the β-casein locus by Cre-mediated site-specific recombination. *Gene* **227**: 21–31.

Krimpenfort P, Rademakers A, Eyestone W, Van Der Schans A, Van Den Broek S, Kooiman P, et al., (1991) Generation of transgenic dairy cattle using '*in vitro*' embryo production. *Bio/Technol* **9**: 844–847.

Kunita R, Samarut J and Pain B (1998) Establishment of the chicken gene-targeting disruption system. French Japonese Workshop. Genes and early development, June 4–5.

Latham PW (1999) Therapeutic peptides revisited nature. *Nature Biotech* **17**: 755–757.

Lee WK, Kim SJ, Hong S, Lee T, Han Y, Yoo OJ, Im KS and Lee K (1998) Expression of a bovine β-casein/human lysozyme fusion gene in the mammary gland of transgenic mice. *J Biochem Mol Biol* **31**: 413–417.

Li X, Eastman EM, Schwartz RJ and Draghia-Akli R (1999) Synthetic muscle promoters: activities exceeding naturally occurring regulatory sequences. *Nature Biotech* **17**: 241–245.

Limonta J, Pedraza A, Rodriguez A, Freyre FM, Barral AM, Castro FO, et al., (1995) Production of active anti-CD6 mouse/human chimeric antibodies in the milk of transgenic mice. *Immunotech* **1**: 107–113.

Litscher ES, Liu C, Echelard Y and Wassarman PM (1999) *Zona pellucida* glycoprotein mZP3 produced in milk of transgenic mice is active as a sperm receptor, but can be lethal to newborns. *Transgenic Res* **8**: 361–369.

Lo D, Pursel V, Linton PJ, Sandgren E, Behringer R, Rexroad C, et al., (1991) Expression of mouse IgA by transgenic mice, pigs and sheep. *Eur J Immunol* **21**: 1001–1006.

Massoud M, Bischoff R, Dalemans W, Pointu H, Attal J, Schultz H, et al., (1991) Expression of active recombinant human alpha 1-antitrypsin in transgenic rabbits. *J Biotech* **18**: 193–204.

Massoud M, Attal J, Thépot D, Pointu H, Stinnakre MG, Theron MC, et al., (1996) The deleterious effects of human erythropoietin gene driven by the rabbit whey acidic protein gene promoter in transgenic rabbits. *Reprod Nutr Dev* **36**: 555–563.

McClenaghan M, Springbett A, Wallace RM, Wilde CJ and Clark J (1995) Secretory proteins compete for production in the mammary gland of transgenic mice. *Biochem J* **310**: 637–641.

McLaren A (2000) Establishment of the germ cell lineage in mammals. *J Cell Physiol* **182**: 141–143.

Meade H (1999) Taking ATIII from goats through clinical trials. *Transgenic Animal Research Conference Tahoe City USA* 33.

Mendez MJ, Green LL, Corvalan JRF, Jia X, Maynard-Currie CE, Yang X, et al., (1997) Functional transplant of megabase human immunoglobulin loci recapitulates human antibody response in mice. *Nature Genet* **15**: 146–156.

Mercier JC and Vilotte JL (1997) The modification of milk protein composition through transgenesis: progress and problems. In: Houdebine LM (ed.) *Transgenic Animals Generation and Use*. (pp. 473–482). Harwood Academic Publishers, Amsterdam.

Mueller S, Prelle K, Rieger N, Petznek H, Lassnig C, Luksch U, Aigner B, Baetscher M, Wolf E, Mueller M and Brem G (1999) *Mol Reprod Dev* **54**: 244–225

Nagaraju J, Kanda T, Yukuhiro K, Chavancy G, Tamura T and Couble P (1996) Attempt at transgenesis of the silkworm (*Bombyx mori* L.) by egg-injection of foreign DNA. *Appl Entomol Zool* **31**: 458–596.

Naito M (1997) The microinjection of DNA into early chicken embryo. In: Houdebine LM (ed.) *Transgenic Animals: Generation and Use*. (pp. 69–73). Harwood Academic Publishers, Amsterdam.

Niemann H, Halter R, Espanion G, Wrenzycki C, Herrmann D, Lemme E, et al., (1996) Expression of human blood clotting factor VIII (FVIII) constructs in the mammary gland of transgenic mice and sheep. *J Anim Breed Genet* **113**: 437–444.

Ninomiya T, Hirabayashi M, Sagara J and Yuki A (1994) Functions of milk protein gene 5′ flanking regions on human growth hormone gene. *Mol Reprod Dev* **37**: 276–283.

Oh KB, Choi YH, Kang Y, Choi WS, Kim MO, Lee KS, Lee KK and Lee CS (1999) A hybrid bovine β-casein/bGH gene directs transgene expression to the lung and mammary gland of transgenic mice. *Transgenic Res* **8**: 307–311.

Page RL, Canseco RS, Russell CG, Johnson JL, Velander WH and Gwazdauskas FC (1995) Transgene detection during early murine embryonic development after pronuclear microinjection. *Transgenic Res* **4**: 12–17.

Paleyanda RK, Velander WH, Lee TK, Scandella DH, Gwazdauskas FC, Knight JW, et al., (1997) Transgenic pigs produce functional human factor VIII in milk. *Nature Biotech* **15**: 971–975.

Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC and Evans RM (1982) Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature* **300**: 611–615.

Palmiter RD, Sandgren EP, Avarbock MR, Allen DD and Brinster RL (1991) Heterologous introns can enhance expression of transgenes in mice. *Proc Natl Acad Sci USA* **88**: 478–482.

Perry ACF, Wakayama T, Kishikawa H, Kasai T, Okabe M, Toyoda Y and Yanagimachi R (1999) Mammalian transgenesis by intracytoplasmic sperm injection. *Science* **284**: 1180–1183.

Petitclerc D, Attal J, Théron MC, Bearzotti M, Bolifraud P, Kann G, et al., (1995) The effect of various introns and transcription terminators on the efficiency of expression vectors in various cultured cell lines and in the mammary gland of transgenic mice. *J Biotechnol* **40**: 169–178.

Pinkaart MJ, Recillas-Targa F and Felsenfeld G (1998) Loss of transcriptional activity of a transgene is accompanied by DNA methylation and histone deacetylation and is prevented by insulators. *Genes Dev* **12**: 2852–2862.

Platenburg GJ, Kootwijk EP, Kooiman PM, Woloshuk SL, Nuijens JH, Krimpenfort PJ, et al., (1994) Expression of human lactoferrin in milk of transgenic mice. *Transgenic Res* **3**: 99–108.

Prieto PA, Mukerji P, Kelder B, Erney R, Gonzalez D, Yun JS, et al., (1995) Remodeling of mouse milk glycoconjugates by transgenic expression of a human glycosyltransferase. *J Biol Chem* **270**: 29515–29519.

Prunkard D, Cottingham I, Garner I, Bruce S, Dalrymple M, Lasser G, et al., (1996) High-level expression of recombinant human fibrinogen in the milk of transgenic mice. *Nature Biotech* **14**: 867–871.

Qureschi SA, Kim RM, Konteatis Z, Biazzo DE, Motamedi H, Rodrigues R, et al., (1999) Mimicry of erythoropoietin by a nonpeptide molecule. *Proc Natl Acad Sci USA* **96**: 12156–12161.

Recillas-Targa F, Bell AC and Felsenfeld G (1999) Positional enhancer-blocking activity of the chicken β-globin insulator in transiently transfected cells. *Proc Natl Acad Sci USA* **96**: 14354–14359.

Reddy VB, Vitale JA, Wei C, Montoya-Zavala M, Stice SL, Balise J and Robl JM (1991) Expression of human growth hormone in the milk of transgenic mice. *Animal Biotech* **2**: 15–29.

Rijnkels M, Miller W and Rosen JM (1999) Casein Gene locus control regions? *Genet Anal Bio-mol Eng* (in press).

Robl JM (1999) New life for sperm-mediated transgenesis? *Nature Biotech* **17**: 636–637.

Rokkones E, Fromm SH, Kareem BN, Klungland H, Olstad OK, Hogset A, et al., (1995) Human parathyroid hormone as a secretory peptide in milk of transgenic mice. *J Cell Biochem* **59**: 168–176.

Ronfort C, Legras C and Verdier G (1997) The use of retroviral vectors for gene transfer into bird embryo. In: Houdebine LM (ed.) *Transgenic Animals: Generation and Use*. (pp. 83–94). Harwood Academic Publishers, Amsterdam.

Rosen JM, Li S, Raught B and Hadsell D (1996) The mammary gland as a bioreactor: factors regulating the efficient expression of milk protein-based transgenes. *Am J Clin Nutr* **63**: 627S–632S.

Rucker EB and Piedrahita JA (1997) Cre-mediated recombination at the murine whey acidic protein (mWAP) locus. *Mol Reprod Dev* **48**: 324–331.

Rudolph NS (1999) Biopharmaceutical production in transgenic livestock. *Trends Biotech* **17**: 367–374.

Saif LJ and Wheeler MB (1998) WAPping gastroenteritis with transgenic antibodies. *Nature Biotech* **16**: 334–335.

Santoso B, Ortiz BD, Winoto A (2000) Control of organ-specific demethylation by an element of the T-cell receptor- locus control region. *J Biol Chem* **275**: 1952–1958.

Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, et al., (1997) Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science* **278**: 2131–2133.

Shamay A, Pursel VG, Wilkinson E, Wall RJ and Hennighausen L (1992) Expression of the whey acidic protein in transgenic pigs impairs mammary development. *Transgenic Res* **1**: 124–132.

Sharma A, Martin MJ, Okabe JF, Truglio RA, Dhanjal NK, Logan JS and Kumar R (1994) An isologous porcine promoter permits high level expression of human hemoglobin in transgenic swine. *Bio/Technol* **12**: 55–59.

Sherman A, Dawson A, Mather C, Gihooley H, Mitchell R, Finnegan D and Sang H (1998) Transposition of the drosophila element mariner into the chicken germ line. *Nature Biotech* **16**: 1050–1053.

Simons JP, McClenaghan M and Clark AJ (1987) Alteration of the quality of milk by expression of sheep beta-lactoglobulin in transgenic mice. *Nature* **328**: 530–532.

Sippel AE, Saueressig H, Huber MC, Faust N and Bonifer C (1997) Insulation of transgenes from chromosomal position effects. In: Houdebine LM (ed.) *Transgenic Animals: Generation and Use*. (pp. 257–265). Harwood Academic Publishers, Amsterdam.

Sohn BH, Kim SJ, Park H, Park SK, Lee SC, Hong HJ, et al., (1999) Expression and characterization of bioactive human thrombopoietin in the milk of transgenic mice. *DNA Cell Biol* **18**: 845–852.

Sola I, Castilla J, Pintado B, Sanchez-Morgado JM, Whitelaw CBA, Clark AJ and Enjuanes L (1998) Transgenic mice secreting coronavirus neutralizing antibodies into the milk. *J Virol* **72**: 3762–3772.

Somia NV, Kafri T and Verma IM (1999) Piecing together more efficient gene expression. *Nature Biotech* **17**: 224–245.

Soulier S, Stinnakre MG, Lepourry L, Mercier JC, Vilotte JL (1999) Use of doxycycline-controlled gene expression to reversibly alter milk-protein composition in transgenic mice. *Eur J Biochem* **260**: 533–539.

Stinnakre MG, Devinoy E, Thépot D, Chêne N, Bayat-Samardi M, Grabowski H and Houdebine LM (1992) Quantitative collection of milk and active recombinant proteins from the mammary glands of transgenic mice. *Anim Biotech* **3**: 245–255.

Stinnakre MG, Soulier S, Schibler L, Lepourry L, Mercier JC and Vilotte JL (1999) Position-independent and copy number related expression of a goat bacterial artificial chromosome α-lactalbumin gene in transgenic mice. *Biochem J* **339**: 33–36.

Strömqvist M, Törnell J, Edlund M, Edlund A, Johansson T, Lindgren K, et al., (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.

Strömqvist M, Houdebine LM, Andersson J, Edlund A, Johansson T, Viglietta C, et al., (1997) Recombinant human extracellular

superoxide dismutase produced in milk of transgenic rabbits. *Transgenic Res* **6**: 271–278.

Taboit-Dameron F, Malassagne B, Viglietta C, Puissant C, Leroux-Coyau M, Chereau C, et al., (1999) Association of the 5′HS4 sequence of the chicken beta-globin locus control region with human EF1 alpha gene promoter induces ubiquitous and high expresssion of human CD55 and CD59 cDNAs in transgenic rabbits. *Transgenic Res* **8**: 223–235.

Tamura T, Thibert C, Royer C, Kanda T, Abraham E, Kamba M, et al., (1999) Germline transformation of the silkworm *Bombyx mori* L. using a *piggyBac* transposon-derived vector. *Nature Biotech* **18**: 81–84.

Thépot D, Devinoy E, Fontaine ML, Stinnakre MG, Massoud M, Kann G and Houdebine LM (1995) Rabbit whey acidic protein gene upstream region controls high-level expression of bovine growth hormone in the mammary gland of transgenic mice. *Mol Reprod Dev* **42**: 261–267.

Tojo H, Tanaka S, Matsuzawa A, Takahashi M and Tachi C (1993) Production and characterization of transgenic mice expressing a hGH fusion gene driven by the promoter of mouse whey acidic protein (mWAP) putatively specific to mammary gland. *J Reprod Dev* **39**: 145–155.

Tomizuka K, Yoshida H, Uejima H, Kugoh H, Sato K, Ohguma A, et al., (1997) Functional expression and germline transmission of a human chromosome fragment in chimaeric mice. *Nature Genetics* **16**: 133–143.

Travers A (1999) Chromatin modification by DNA tracking. *Proc Natl Acad Sci* **96**: 13634–13637.

Van Cott KE, Williams B, Velander WH, Gwazdauskas F, Lee T, Lubon H, Drohan WN (1996) Affinity purification of biologically active and inactive forms of recombinant human protein C produced in porcine mammary gland. *J Mol Recognit* **9**: 407–414.

Verrinder-Gibbins AM (1998) The chicken, the egg, and the ancient mariner. *Nature Biotech* **16**: 1013–1014.

Vos JH (1998) Mammalian artificial chromosomes as tools for gene therapy. *Curr Opin Genet Dev* **8**: 351–359.

Wagner K, Wall RJ, St Onge L, Gruss P, Wynshaw-Boris A, Garrett L, et al., (1997) Cre-mediated gene deletion in the mammary gland. *Nucleic Acids Res* **25**: 4323–4330.

Wall RJ (1999) Biotechnology for the production of modified and innovative animal products: transgenic livestock bioreactors. *Lives Prod Sci* **59**: 243–255.

Walters MC, Magis W, Fiering S, Eidemiller, Scalzo D, Groudine M and Martin DIK (1996) Transcriptional enhancers act in *cis* to suppress position-effect variegation. *Genes Dev* **10**: 185–195.

Wang Y, DeMayo FJ, Tsai SY and O'Malley BW (1996) Ligand-inducible and liver-specific target gene expression in transgenic mice. *Nature Biotech* **15**: 239–243.

Weidle UH, Lenz H and Brem G (1991) Genes encoding a mouse monoclonal antibody are expressed in transgenic mice, rabbits and pigs. *Gene* **98**: 185–191.

Wen J, Kawamata Y, Tojo H, Tanaka S and Tachi C (1995) Expression of whey acidic protein (WAP) genes in tissues other than the mammary gland in normal and transgenic mice expressing mWAP/hGH fusion gene. *Mol Reprod Dev* **41**: 399–406.

Wheeler M (1999) Transgenic alteration of sow milk: production and characterization bovine a-lactalbumin and IGF-I transgenic swine. *Transgenic Animal Research Conference Tahoe City USA*, 28–29.

Whitelaw B, Harris S, McClenaghan M and Simons JP (1992) Position-independent expression of the ovine β-lactoglobulin gene in transgenic mice. *Biochem J* **286**: 31–39.

Whitelaw B (1999) Toward designer milk. *Nature Biotech* **17**: 135–136.

Willard FH (1998) Human artificial chromosomes coming into focus. *Nature Biotech* **16**: 415–416.

Wright G and Colman A (1997) Purification of recombinant proteins from sheep's milk. In: Houdebine LM (ed.) *Transgenic Animals: Generation and Use.* (pp. 469–471). Harwood Academic Publishers, Amsterdam.

Wright A and Morrison SL (1997) Effect of glycosylation on antibody function: implications for genetic engineering. *Trends Biotech* **15**: 26–32.

Wright G, Carver A, Cottom D, Reeves D, Scott A, Simons P, et al., (1991) High level expression of active human alpha-1-antitrypsin in the milk of transgenic sheep. *Bio/Techno* **9**: 830–834.

Wrighton NC, Farrell FX, Chang R, Kashyap AK, Barbone FP, Mulcahy LS, et al., (1996) Small peptides as potent mimetics of the protein hormone erythropoietin. *Science* **273**: 458–464.

Yamao M, Katayama N, Nakazawa H, Yamakaws M, Hayashi Y, Hara S, et al., (1999) Gene targeting in the silkworm by use of a baculovirus. *Genes Dev* **13**: 511–516.

Yarus S, Rosen JM, Cole AM and Diamond G (1996) Production of active bovine tracheal antimicrobial peptide in milk of transgenic mice. *Proc Natl Acad Sci USA* **93**: 14118–14121.

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

Zinovieva N, Lassnig C, Schams D, Besenfelder U, Wolf E, Müller S, et al., (1998) Stable production of human insulin-like growth factor 1 (IGF-1) in the milk of hemi- and homozygous transgenic rabbits over several generations. *Transgenic Res* **7**: 437–447.

Zuelke KA (1998) Transgenic modification of cows milk for value-added processing. *Reprod Fertil Dev* **10**: 671–676.