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Production of pharmaceutical proteins in milk

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Abstract. There is every reason to expect that it will be possible within the next few years to begin to use farm animals to produce large quantities of some of the human proteins that are needed for the treatment of disease. Revolutionary new opportunities for the production of novel proteins in milk have been created by the development of methods for gene transfer. Exploitation of these opportunities depends upon selection and cloning of milk protein genes and identification of the sequences that govern tissue specific hormonally induced expression in the mammary gland. Studies with three genes, ovine β-lactoglobulin, rat β-casein and whey acidic protein of rat and mouse, suggest that they may all meet this requirement. Fragments of the ovine β-lactoglobulin, murine whey acidic protein and rabbit β-casein genes have directed production of novel proteins in the milk of transgenic mice, sheep, rabbits and pigs. The proteins were biologically active and usually co-migrated with authentic proteins. In early experiments, protein concentration was low, but our recent observations suggest that fusion genes containing genomic clones direct production of concentrations of protein that are suitable for commercial exploitation.

In the longer term, two approaches may offer the potential of more reliable expression. Control elements capable of directing expression that is independent of site of insertion of the gene, but dependent on the number of copies of the gene, have been identified for a small number of genes. The availability of such elements for the milk protein genes would increase the reliability of gene expression considerably. Alternatively, targeted mutation of genes may allow the insertion of coding sequences within an existing gene so avoiding position effects.

Key words. Gene transfer; gene modification; gene expression; livestock; transgenic animal; pharmaceutical proteins; milk composition.

Introduction

The ability to transfer genes into the germline of livestock creates revolutionary new opportunities for the modification of animal production traits, including the composition of milk. The mammary gland of a dairy animal can be envisaged as an efficient vat for the production of specific proteins, sugars and fats. In this role it has long been exploited by Man as a source of food, particularly protein. Changes may be envisaged either to proteins that are normally produced in milk[48] or to direct production of novel proteins to the mammary gland[26].

This paper will review methods of genetic manipulation and experiments to target gene expression to the mammary gland, before discussing potential applications of the technology for the production of pharmaceutical proteins in milk. Particular emphasis will be placed upon the use of techniques for gene transfer as these are available at present, but mention will also be made of targeted mutation.

Methods of genetic manipulation

Detailed reviews have been published elsewhere of the procedures for genetic manipulation in livestock, of their limitations and of the different approaches that are being taken to establish more effective techniques [38, 49]. In this review it is only necessary to appreciate the limitations to the present techniques and the greater opportunities that may become available.

Genes have been transferred into the germline of pigs, sheep and cattle by direct injection of several hundred copies of the gene into a nucleus in the early embryo [38]. This procedure has been used successfully in several different laboratories, but has a number of disadvantages. First, the proportion of injected eggs that survive to become live transgenic offspring is small, being around 1%. It seems that the proportion is highest in the pig and lowest in the cow, but this apparent difference may be directly related to differences between species in the number of eggs that have so far been injected and transferred (pig > sheep > cow). Clearly the cost of producing transgenic livestock with current procedures is high as there is a requirement for large numbers of donor and recipient females. Secondly, the site of integration is apparently random and insertion of the gene is associated with mutation of an endogenous gene in some cases. It is suggested that the act of injecting fluid into the nucleus causes breaks in the chromosomes and that at the time of repair the injected gene is inadvertently included in the chromosome [34]. The gene is commonly inserted in a tandem array and there may be rearrangements at the site of integration. Finally, there are very marked differences between lines in the pattern of expression of the transgene. These differences are believed to reflect an influence of neighbouring DNA upon the transgene and are discussed in detail later.

Many of these limitations would be overcome by the development of site directed methods of gene insertion. Site directed changes have been introduced in mice by homologous recombination in embryonic stem cells [13]. Typically the vectors that have been used have long sequences of homology with the endogenous sequences, but only a short divergent region. So far they have been used to make small changes to endogenous DNA sequences, usually to disrupt gene expression. Recently a two-step method has been described which may be used to make small changes in the DNA sequences [45]. Before site directed methods of gene insertion can be used in the production of pharmaceutical proteins in livestock it will be necessary to enhance the procedures for homologous recombination to allow the introduction of long coding regions and to isolate embryonic stem cells from these species. Recent reports of the maintenance in culture of pig, sheep and cattle cells that resemble mouse embryonic stem cells suggests that at least this latter requirement may be met quite soon [33].

Targeting expression to the mammary gland

The milk proteins and the nature of milk

The mammary gland has been selected as a site for the production of novel proteins partly because at the peak of lactation it secretes substantial quantities of protein into the milk: around 0.1 and 1 kg per day in sheep and cattle respectively. Most of the protein is synthesised by the mammary epithelium, but some blood serum proteins, such as albumin, are present in milk in smaller quantities. The table lists the major proteins of cow, sheep, mouse and human milk.

Caseins, a family of phosphoproteins, are the major milk proteins and contribute over 80% of milk protein. Caseins and genes encoding caseins from a number of species have been characterised and these studies reveal considerable species differences in protein and DNA sequences [39]; they are however very similar in their biophysical properties. Milk is a colloid and it is interactions between casein molecules and calcium phosphate that lead to the formation of micelles. In the presence of physiological levels of calcium, (bovine) α_{S1}-, α_{S2}- and β-caseins aggregate. The size of the aggregates is limited by κ-casein, which forms a coat around the micelle and prevents further aggregation. This casein has only one phosphoserine, as compared to 5–11 in the other caseins, and as a result is soluble in the presence of high concen-

Protein composition of milk from a number of species (data taken from Lathe et al. [26])

	Concentration in milk (g/l)			
	Cow	Sheep	Mouse	Human
Caseins				
α_{S1}-casein	10	12	*	0.4
α_{S2}-casein	3.4	3.8	*	*
β-casein	10	16	*	3
κ-casein	3.9	4.6	*	1
Major whey proteins				
α-lactalbumin	1	0.8	Trace	1.6
β-lactalbumin	3	2.8	None	None
Whey acidic protein	None	None	2	None
Other whey proteins				
Serum albumin	0.4	*	*	0.4
Lysozyme	Trace	*	*	0.4
Lactoferrin	0.1	*	*	1.4
Immunoglobulins	0.7	*	*	1.4

* No data available.

trations of calcium. It is also the only casein that is glycosylated and polarised. The hydrophobic regions of the κ-casein molecules are believed to interact with the other caseins while the hydrophilic regions interact with the aqueous phase of milk. The structure of caseins and their role in micelle formation are reviewed by Kang et al.[24] and Jimenez-Flores and Richardson[23]. κ-casein is the substrate for the protease rennin; cleavage of κ-casein destabilises the micelles and results in precipitation of the other caseins, forming the curds. This property of milk is exploited in cheese making. Precipitation of caseins also results from exposure of milk to low pH. Stability of the micelles during milk processing is influenced by the relative concentrations of κ-casein and β-lactoglobulin. Addition of κ-casein to milk increases the tolerance to heating. Casein genes have been cloned and used to direct production of proteins to milk (see below).

A large number of proteins are found in whey, but there are differences between species in the nature of the whey proteins. The major whey proteins of ruminants are β-lactoglobulin and α-lactalbumin. In rodents α-lactalbumin and whey acidic protein are the major proteins and β-lactoglobulin is absent. These whey proteins are synthesised in the mammary epithelium, while many others enter milk from the bloodstream. In ruminants, the most abundant whey protein is β-lactoglobulin, which is present at about 3 g/l in cows and sheep; in sheep approximately 5% of poly-A^+RNA encodes β-lactoglobulin[31]. The protein is an 18 kDa dimer which may function in the transport of retinol to the young[35]. The ovine gene has been cloned[1] and is the subject of detailed studies (see below).

Whey acidic protein, present only in rodents and rabbits has no known function. It has a mass of 14 kDa and has some structural similarities and a similar distribution of cysteine residues to a family of proteins that includes neurophysin, snake venom toxin, wheat germ agglutinin and rag weed allergen Ra5[22]. It was suggested that this similarity may indicate that the proteins have a similar three-dimensional structure. The gene has been cloned and is the subject of studies to direct production of novel proteins to the mammary gland (see below).

When contemplating modifications to the proteins in milk, it is important to recognise that changes to the amount or structure of one component may have profound effects upon the nature of the milk because of the complex interactions between the constituents. In particular, changes to proteins may affect micelle structure and so change the milk dramatically. When using dairy animals to synthesise specific proteins for extraction and pharmaceutical or industrial use, this is not likely to be important. However, when the milk is for human consumption, any changes that make processing more difficult would be extremely inconvenient. There is also a need to consider the well-being of neonates that at some time in the future may obtain the modified milk from their mothers.

Regulation of milk protein gene expression

There are considerable differences between milk proteins in the pattern of accumulation of their individual mRNAs during gestation and lactation[39]. In the gland of the virgin rat there are several hundred α-casein mRNA molecules per cell, but very few whey acidic protein mRNA molecules. By mid-gestation, the level of β-casein has increased more rapidly than the other mRNA molecules and it has become the most abundant species, a position it retains throughout pregnancy and lactation. These observations establish that in addition to the mechanisms to stimulate cell division and promote the development of the mammary gland, there are mechanisms which regulate expression of the individual milk protein genes.

Analysis of the control of gene expression in the mammary gland has been handicapped by the absence of cell lines that retain specific milk protein synthesis and respond to hormones. It has become clear that control in mammary gland is particularly complex and involves interactions between cells, as well as between cells and the extra-cellular matrix and hormonally induced factors[7,39]. Transgenic animals allow the study of expression of transferred genes in the native mammary gland, with all the normal developmental and hormonal signals. To date, studies in transgenic animals have been published with five milk protein genes; ovine β-lactoglobulin, murine whey acidic protein, bovine α-lactalbumin, rat β-casein and bovine α-casein. These observations will be reviewed before considering the potential application of targeting gene expression to the mammary gland.

Beta-lactoglobulin

The Edinburgh group selected β-lactoglobulin for study as it is the whey protein present at highest concentration in ruminant milk. Four clones were isolated from a genomic library of a sheep, one of which (clone SS1) has been characterised in detail[1,20]. The sequence of SS1 revealed that the β-lactoglobulin gene has seven exons. In order to discover whether the clone contained all of the cis-acting sequences necessary for efficient tissue specific expression of the gene, the clone was introduced into mice[43]. Nine transgenic mice were selected for study; five of the mice were female, milk was collected from them and analysed for the presence of β-lactoglobulin. Three of these animals produced β-lactoglobulin, the two which failed to do so carried less than one copy of the gene per cell. Transgenic lines were established from two of the expressing females and from three males. In four of the five lines, females secrete β-lactoglobulin into their milk. In the other line, it is inferred that the gene was incorporated into the Y chromosome since all transgenic mice of this line are male. When studied by SDS polyacrylamide electrophoresis, the β-lactoglobulin in mouse milk co-migrated with that in sheep milk and with purified β-lactoglobulin. In the most abundantly expressing line, the concentration of β-lactoglobulin was 23 mg/ml, five

times that in the control sheep milk. The pattern of expression of the transgene was analysed in three lines by Northern blotting of RNA prepared from mammary, salivary and lachrymal glands, liver, kidney and spleen. Abundant transcripts were found only in mammary gland. Production of this sheep protein in mouse milk had no apparent side-effects upon the mothers or the young.

The developmental regulation of the transgene has been studied in two lines of mice [21]. The changes in the level of expression of β-lactoglobulin paralleled that of the endogenous β-casein gene. This pattern is similar to that of β-lactoglobulin in sheep, but different from the endogenous mouse whey acidic protein. These observations show that clone SS1 contains all of the cis-acting sequences that are essential for high level tissue-specific expression of the gene. In addition, it seems that these sheep sequences can be interpreted correctly by the mammary gland of mice despite the fact that β-lactoglobulin is absent from mice and that there are differences between mice and sheep in the hormonal regulation of lactation. In subsequent experiments, the nature of the cis-acting sequences is being studied by deletion of 5′ sequences before gene transfer. Preliminary results suggest that all of the essential 5′ sequences lie within 0.8 kb of the start site [21].

This gene and fragments of it have been used to direct production of human therapeutic proteins to the mammary gland. The proteins chosen for expression are clotting factor IX and α_1-antitrypsin (also known as α_1-protease inhibitor). In the first experiments, the entire β-lactoglobulin gene was used, contained in a fragment of 10.5 kb which functions well in transgenic mice [43]. A fragment from a human factor IX cDNA clone was inserted into the 5′ untranslated region of the β-lactoglobulin clone [16]. The human sequences encode the entire pre-pro-protein except for a rearrangement of the first AUG of the RNA; they have been used successfully for the production of biologically active factor IX from cells in tissue culture [4]. At the 5′ junction the sheep and human sequences were fused in their 5′ untranslated regions, while the human fragment includes a stop codon, but no polyadenylation site. The predicted fully processed transcript would contain the coding sequences for both proteins with the factor IX termination codon preceding the β-lactoglobulin initiation codon by 167 nucleotides. Four transgenic sheep were produced by microinjection of the β-lactoglobulin-factor IX hybrid gene into one-cell eggs [44].

Two of the transgenic sheep were female and their milk has been examined for the presence of factor IX [16]. Radioimmunoassay of freeze-dried whole milk samples showed the milk of both ewes to contain approximately 25 ng/ml factor IX. This is around 1/250th the concentration in human plasma and 1/100,000th the concentration of β-lactoglobulin in sheep milk. The factor IX was enriched from the milk by monoclonal antibody affinity chromatography and was shown to be active in a clotting assay. The steady-state level of the fusion gene RNA was found to be approximately 1/1250th that of the endogenous β-lactoglobulin mRNA. A similar construct containing α_1-antitrypsin cDNA in place of the factor IX sequences has also been introduced into sheep and expression has been studied in two ewes. One ewe produced α_1-antitrypsin at a considerably greater concentration than had been observed with factor IX (approximately 5 μg/ml). While this compares favourably with the expression of factor IX, the level of β-lactoglobulin is about 1000 times greater than that of human α_1-antitrypsin in the milk of this animal.

The level of expression of these first fusion genes was too low to be of commercial value and in order to optimise expression, a number of new fusion genes are being studied in mice. One fusion gene directs considerably higher levels of α_1-antitrypsin production than the first construct [5]. This gene contains only 5′ sequences of β-lactoglobulin and a genomic fragment of α_1-antitrypsin. There were three different patterns of expression of the transgene. In 13 independent mice or mouse lines, 5 expressed the transgene in the mammary gland, 5 in the salivary gland and in 2 the gene was expressed in both tissues. No expression was observed in the other line. Four of the five lines in which expression was observed in the mammary gland produced concentrations over 0.5 mg/ml and one of these produced 7 mg/ml. The higher concentration of protein in this experiment adds to the evidence that introns may have significant effects upon gene expression in transgenic animals [8].

Whey acidic protein

Whey acidic protein is the whey protein present at greatest concentration in mouse and rat milk. The protein is encoded by a 2.8 kb gene having four exons [12]. Genomic clones of the mouse gene have been transferred to mice and pigs [11, 47] and 5′ flanking sequences have been used to direct tissue-specific hormone-dependent expression of two oncogenes [2, 3] and of human tissue plasminogen activator in mice [18, 36]. A genomic clone of the rat gene has also been transferred to mice [6]. There is evidence from these studies that expression of the whey acidic protein gene is inhibited at some stages of pregnancy and that removal of this inhibition may enhance expression of the gene.

The mouse clone that has been studied in pigs and mice was a genomic clone with 2.6 kb of 5′ sequences and 1.6 kb of 3′ flanking DNA. The concentration of whey acidic protein in the milk was around 1 mg/ml in all three pig lines studied, which is approximately the same concentration of the protein as is found in mouse milk [47]. While the observations are only based upon three lines, and therefore should be treated with caution, it seems possible that in pigs the expression of the gene is relatively independent of the position effect. By contrast, when the same gene was transferred to mice the variation in

level of expression was comparable to that with other transgenes[11]. The rat genomic clone carried 0.949 kb of 5′ flanking DNA and 1.4 kb of 3′ sequences[6]. The protein was present in the milk in 8 of 9 mouse lines at an average concentration of 27% of the endogenous mouse protein (range from 1% to 95%). Whey acidic protein is unusual in that the increase in the mRNA concentration occurs later in pregnancy than for other milk proteins and it is interesting to note that the increase in transcription of the rat transgene occurred earlier than that of the endogenous mouse gene, suggesting that the clone transferred had escaped an inhibitory mechanism.
A 2.6 kb fragment containing the promoter and 5′ sequences of the mouse gene has been shown to direct tissue-specific hormone-dependent expression of two oncogenes[2,3] and of human tissue plasminogen activator[18,36] in transgenic mice. The fusion genes contained 2.6 kb of 5′ sequences terminating 24 bp downstream of the transcription start site. This fragment contains binding sites for proteins that are specific to lactating mammary gland and which may be cis-acting regulatory elements[28]. These sequences were capable of directing tissue-specific expression of human Ha-*ras* and murine c-*myc* gene expression to the mammary gland of transgenic mice[2,3]. The level of RNA of the two fusion genes was appreciably lower than that of the endogenous whey acidic protein gene (between 2% and 10%), but followed similar profiles during lactation. In both cases, expression of the fusion gene was marked by the development of mammary tumours, although the speed of development was more rapid and the incidence more frequent with the *myc* construct. Tumour formation in these mice was dependent on them having gone through lactation. Interestingly, in the tumours with the *myc* oncogene, expression of the endogenous milk protein genes was independent of hormonal stimulation[41]. In animals with the whey acidic protein-*myc* construct, endogenous whey acidic protein and the transgene were only expressed during lactation in normal mammary tissue. By contrast, in tumours from the same mice, whey acidic protein and β-casein genes were expressed after lactation and following transfer to nude mice.
The same 5′ whey acidic protein sequences have been used to direct expression of human tissue plasminogen activator in the mammary glands of transgenic mice. The whey acidic protein sequences were linked to the 5′ untranslated region of a cDNA coding for tissue plasminogen activator. The plasminogen activator cDNA encoded the entire protein, including its secretion signal sequence. At the 3′ end of the construct, a polyadenylation signal derived from SV40 was incorporated[18]. Milk from six transgenic lines of mice was analysed and four were found to produce biologically active protein. As determined by ELISA the concentration of tissue plasminogen activator was around 50 μg/ml in the line with the greatest concentration[36]. In that line the steady state level of mRNA for the fusion gene was approximately 1% that of the endogenous whey acidic protein RNA. RNase protection assays showed that the transcription start site was the same for the endogenous gene and the transgene. During pregnancy the level of expression of both fusion and endogenous genes was greatly increased in the mammary gland, by approximately 100- and 10,000-fold respectively. The transgene was expressed predominantly in the mammary gland, but low levels of expression were detected in sublingual gland, tongue and kidney. The level of expression of the two genes did not change significantly in other tissues during pregnancy.
A rather shorter fragment of the murine whey acidic protein gene has also been used to drive production of a soluble form of human CD4 protein in transgenic mouse milk[50]. The whey acidic protein gene fragment containing 1.85 kb of 5′ DNA was fused to a partial genomic clone of the human protein gene which encodes a soluble form of the protein. The human protein was present in the milk of 5 of the 7 lines studied at concentrations ranging from 10 to 200 ng/ml. As the protein bound the gp120 envelope glycoprotein of HIV-1 and 2 monoclonal antibodies it is probable that it had a normal conformation.
The 5′ whey acidic protein fragments used in these fusion genes apparently contain some of the sequences necessary for tissue-specific hormonally induced expression, but perhaps not all. The relative concentration of whey acidic protein mRNA increased to a greater extent than that of the fusion gene (1000-fold compared with 100-fold) and at a later stage of gestation (days 14–16 compared to day 10). There may be sequences that govern response to hormone in a more distant 5′ site, in the coding region or the 3′ sequences. It will also be necessary to account for the apparent inhibitory mechanism. Much remains to be learned of the mechanisms that regulate transcription and translation of this gene.

Alpha-lactalbumin

Alpha-lactalbumin is found in the milk of most species and is involved in the synthesis of lactose[30]. The bovine gene has been cloned and a genomic fragment transferred into mice[46]. The clone contained the entire transcription unit, which extends over 2 kb, and 750 and 336 bp of 5′ and 3′ flanking DNA, respectively. Tissue-specific expression was observed in 5 of the 6 lines studied, however the concentration of protein varied considerably. In cattle the concentration is around 1 mg/ml and in two of the transgenic lines the concentrations of 0.25 and 0.45 mg/ml approached this concentration. In the other three lines the concentration was of the order of μg/ml. These observations suggest that the clone transferred had many, if not all of the elements necessary for efficient expression, however the relatively low concentration of the protein in cattle suggest that it may not be the best possible candidate for use in the production of foreign proteins.

Beta-casein

Beta-casein is the milk protein present at highest concentration. A rat genomic clone was isolated and transferred into mice[27]. The gene is 7.5 kb long and has 9 exons, the first and last of which are non-coding[39]. In transgenic mice the gene was expressed in a developmentally regulated and tissue-specific manner, but at only 0.01–1% of the level of the endogenous mouse β-casein gene[27]. An RNase protection assay confirmed that transcription was initiated at the authentic start site. The low level of expression may reflect an effect of site of integration of the transgenes (only three lines were studied) or the fact that important cis-acting regulatory elements lie outside the region cloned and transferred.

Approximately 2 kb of 5′ flanking DNA of the rabbit β-casein has been used to drive secretion of human interleukin-2 to the milk of rabbits[10]. A genomic clone of the interleukin-2 gene was fused to the milk protein gene sequences in their 5′ untranslated regions. Biologically active protein was found in the milk of all four females tested, but the concentration was low, being 50–430 ng/ml respectively.

Alpha-casein

α_{s1}-casein is the major protein of cows milk and accounts for approximately 30% of the protein. A mammary specific vector was constructed with 21 kb of 5′ sequences and 2 kb of 3′ flanking DNA joined by a synthetic linker[29]. A genomic clone of human urokinase was inserted into the vector with the entire coding region and some 1 kb of sequences downstream of the polyadenylation site. Of 3 transgenic mice created only 1 passed on the gene to its offspring. In females of this line the human protein was present in the milk at a concentration of 1–2 mg/ml. The urokinase was biologically active and of the same molecular weight as authentic human protein as judged by polyacrylamide gel electrophoresis. If this level of production could be obtained in other transgenic lines, and particularly in livestock, it would establish the potential of the vector in the production of pharmaceutical proteins in milk.

Production of pharmaceutical proteins

Transgenic farm animals provide one possible route for the large-scale production of proteins that cannot be produced by present commercial procedures. A number of human proteins are now produced by recombinant microorganisms[17,37,40], but there are other proteins that cannot be manufactured in this way because microorganisms are unable to complete the necessary post-translational modifications. At present proteins that require modification, such as clotting factors, are prepared from human or animal material.

Production of therapeutic proteins in the milk of farm animals has several potential advantages. First, substantial quantities of protein are produced by the mammary gland. Second, the protein can be collected frequently, without distress to the animal and in a sterile manner. Third, the protein is isolated from the other tissues of the animal so minimising the risk of undesirable side effects caused by the foreign protein. By contrast, a biologically active protein produced by the liver might well exert its effect upon the producing animal. Finally, preparations from milk would probably be free of contamination by infectious agents such as the agents that cause AIDS and hepatitis. By contrast, preparations of clotting factors from human blood have in the past contained these agents and patients requiring treatment with such proteins have had a high risk of infection. If protein for the treatment of human disease is produced in the milk of farm animals it would be essential to ensure that no other infectious agents were present.

Other potential means of producing these proteins include tissue culture of mammalian cells[14] or of insect cells transfected by bacillovirus[32]. Transgenic animals may ultimately be a cheaper source of recombinant proteins than cells maintained in tissue culture. Lines of transgenic animals are expensive to create, but then cheap to multiply and use. By contrast, large-scale tissue culture of cells will always be difficult and expensive. The relative advantages of these two approaches remain to be determined. There is every reason to expect that production of the proteins could be directed to the blood in transgenic livestock, as for example, factor IX has been produced in the liver of transgenic mice and secreted into blood[15]. However, milk has the advantage that it is very easy to collect and the volume of milk that could be collected would be greater than the available volume of blood. In addition, milk is relatively isolated from the animal. The ease and expense of purification of the product must also be borne in mind. New processes will have to be developed for extraction of proteins from milk, but there is no reason to suspect that this will be more taxing than purification of proteins from other sources such as bacteria.

The therapeutic use of recombinant-DNA derived protein will depend upon production of biologically active material and purification to homogeneity. The experiments performed so far suggest that transgenic animals have produced biologically active clotting factor IX[16], urokinase[29], α_1-antitrypsin[5], tissue plasminogen activator[18], human interleukin-2[10] and a soluble form of human CD4 protein[50] in their milk. Before proteins may be used therapeutically, considerable work will be required to verify their structure and activity, and to demonstrate efficacy.

Three factors are likely to have major effects upon the development of this approach in farm animals. First, it remains to be confirmed that fusion genes can direct production of high concentrations of protein in milk in livestock species and there is a great need to decrease the variability in the concentration of protein produced. Much remains to be learned of the cis-acting elements

that regulate expression of the milk protein genes. Elements have been defined for two genes, the globin gene family and chicken lysozyme, which render expression of transgenes relatively independent of position, but dependent upon number of copies of the transgene[19,42]. The mechanism by which this effect is achieved is not known, but may involve binding of specific transcription factors and/or of the nuclear matrix. The identification of such dominant control regions for milk protein genes would greatly increase the efficiency of projects with transgenic animals, as a far greater proportion of transgenic lines would be expected to produce high concentrations of the protein than with present procedures. Alternatively, the establishment of techniques for the targeted insertion of coding sequences adjacent to the regulatory elements of an endogenous milk protein gene (see section on 'Methods of genetic manipulation') would provide an effective means of avoiding the position effect. In addition, this latter approach could disrupt production of the milk protein. If the amount of protein produced by the mammary gland is limited, then prevention of transcription of a milk protein gene would be expected to slightly increase the yield of the foreign protein. Future experiments will also provide a great deal more information concerning the role of introns in gene expression and provide comparisons between different milk protein genes and different species of dairy animal.

Secondly, much remains to be learned about the ability of the mammary gland to perform post-translational modifications. Biologically active clotting factor IX has been produced at very low levels by a number of cell lines[4,9]. In this case a major limitation seems to arise from the relative inability of the cells to carry out gamma-carboxylation[25]. When larger amounts of protein were produced only a small proportion was biologically active. The potential of the mammary gland in this respect remains to be determined.

Finally, there are observations in transgenic lines with several different transgenes that expression in the mammary gland is associated with disruption of lactation[29,43,47]. Further studies are needed to discover the mechanism behind this effect and to establish precautionary measures.

The principal is established that the mammary gland is able to make some proteins of therapeutic value, but much more remains to be learned. The very high value of the products make this an avenue of development that will be followed up very vigorously. Once established it may then be exploited for the production of other proteins.

Milk proteins

There are several possible means of introducing changes to endogenous milk proteins: by introducing extra copies of existing genes, by using regulatory sequences from a protein gene that is expressed at a higher level, by transferring milk protein genes from another species or by transferring modified genes. There may be benefit in directing the production of human milk proteins to the milk of farm animals in order to make the milk a better substitute for human milk. In particular, it has been suggested that lactoferrin may be important[30]. Milk containing human lactoferrin might be obtained either by transfer of the intact human gene, if its regulatory elements recognise the trans-acting factors of the transgenic animal, or by transfer of a fusion gene in which the human coding sequences were regulated by a milk protein gene of another species.

Conclusions

Methods of gene transfer are very new and still particularly expensive in farm animals. As discussed earlier, improvements in efficiency and reductions in cost are to be expected. However, the great cost of the present procedures will limit their exploitation for some time and initially ensure that they are used for the particularly high value products, such as pharmaceutical proteins. The results obtained in recent years show that there is every reason to expect that it will be possible within the next few years to begin to use farm animals to produce large quantities of some of the human proteins that are needed for the treatment of disease. Experience will be gained from attempting such applications, and in time it seems probable that these techniques will also be used to modify the composition of milk for liquid consumption or food processing.

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