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The Genes Encoding the Major Milk-Specific Proteins and Their Use in Transgenic Studies and Protein Engineering

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Introduction

This chapter begins with the characteristics of the genes encoding the following milk proteins: the caseins, the whey proteins α-lactalbumin, β-lactoglobulin (BLG), whey acidic protein (WAP), late lactation protein, lactoferrin and lysozyme. It then goes on to cover milk protein transgenic studies with specific reference to using the mammary gland as a bioreactor for the production of biologically active proteins and the genetic engineering of milk composition. However, this chapter does not cover aspects of the hormonal regulation of milk protein gene expression, the biosynthesis of milk proteins and the role of the cell–cell and cell–extracellular matrix interactions which have been treated by Rosen *et al.* (1986), Mepham *et al.* (1992) and Lin and Bissell (1993). The milk proteins and their biological function have recently been discussed by Sawyer and Holt (1993), while a comprehensive overview of milk protein gene organization is given by Mercier and Vilotte (1993).

Milk proteins

The primary role of milk is to provide the suckling newborn with all its nutritional requirements for normal growth and development. Milk is a rich source of vitamins,

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Abbreviations: α_1AT, α_1-antitrypsin; bFSH, bovine follicle-stimulating hormone; BLG, β-lactoglobulin; BST, bovine somatotropin; CAT, chloramphenicol acetyltransferase; CHO, Chinese hamster ovary; DCRs, dominant control regions; ELISA, enzyme-linked immunosorbent assay; ES, embryonic stem; FIX, blood clotting factor IX; hCD4, human CD4; hCFTR, human cystic fibrosis transmembrane conductance regulator; hIL-2, human interleukin-2; hSA, human serum albumin; IGF-I, insulin-like growth factor-I; LLP, late lactation protein; MAR, matrix attachment regions; oBLG, ovine β-lactoglobulin; oTP, ovine trophoblast interferon; PCR, polymerase chain reaction; SOD, extracellular superoxide dismutase; t-PA, tissue plasminogen activator; UTR, untranslated region; WAP, whey acidic protein

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minerals, lipids, carbohydrates and amino acids, constituting a complete food source for the neonate. It has been estimated that in the United States, cow's milk still provides approximately 25% of the population's total protein, calcium and riboflavin intake. Therefore, milk remains an important source of nutrition throughout life (Swaisgood, 1973).

The bovine milk proteins are probably the best characterised, chemically, physically and genetically, of all food proteins (Swaisgood, 1993). Milk proteins are divided into two distinct groups. First, the caseins, which are a group of acidic, proline-rich, phosphoproteins that precipitate out of skim milk at pH 4.6 and 20°C (Jollès, 1975). The proteins that remain in solution under these conditions are termed the whey proteins, and these constitute a large class of diverse globular polypeptides. The function of the casein fraction is to chelate colloidal calcium phosphate and so serves as a major source of amino acids, calcium and phosphate for the suckling infant.

The protein composition of the milk of different species varies greatly, both in the presence or absence of particular proteins and in their relative abundance. One or more of the α-caseins, β- and κ-caseins, and α-lactalbumin have been found in the milks of most mammals studied so far (although human milk appears to lack an α-casein).

BLG is the major whey protein of ruminants such as the cow (Braunitzer *et al.*, 1973), sheep (Ali and Clarke, 1988) and red deer (McDougall and Stewart, 1976). It is also present in the milk of many non-ruminant species, such as the pig and horse (Godovac-Zimmerman and Braunitzer, 1987), dog, dolphin and manatee (Pervaiz and Brew, 1986), kangaroo (Godavac-Zimmerman and Shaw, 1987), whale (Ullrey *et al.*, 1984), tammar wallaby (Collet, Joseph and Nicholas, 1991), monkey (Azuma and Yamauchi, 1991), cat (Halliday *et al.*, 1990) and possibly the echidna and platypus (Teahan, McKenzie and Griffiths, 1991) but is absent from the milk of mice, rats and humans (Pervaiz and Brew, 1985). BLG has been implicated in a variety of activities, including the transport of retinol in milk (Papiz *et al.*, 1986), to aiding the digestion of milk lipids through binding and so removing the fatty acids which inhibit pregastric lipase (Perez *et al.*, 1992).

WAP is the major whey protein in the mouse (Hennighausen and Sippel, 1982a), rat (Hobbs *et al.*, 1982) and rabbit (Devinoy *et al.*, 1988). WAP may interact with an unknown receptor in either the digestive system or the mammary gland, may carry trace metals or may simply have a nutritional role as a source of sulphur (Hennighausen and Sippel, 1982b).

Lactoferrin is an iron-binding glycoprotein and has a variety of functions, including inhibiting microbial growth (Arnold, Cole and McGhee, 1977), stimulating the immune system (Sawatzki and Rich, 1989) and aiding iron absorption in infants (Nemet and Simonovitz, 1985). Lactoferrin has been found in the milk of a number of species, including the guinea-pig, cow, goat, pig, mare, mouse (Masson and Heremans, 1971), and the human (Johansson, 1960).

Lysozyme is believed to be present in the milk of most mammals (Steinhoff, Senft and Seyfert, 1994). The low concentration in cow's milk (0.5 μg ml^{-1}) when compared to human milk (500 μg ml^{-1}) (Meyer, Erhard and Senft, 1981), prompted the suggestion that these low levels may derive from blood cells in the mammary gland (Steinhoff, Senft and Seyfert, 1994). There appear to be at least 10 copies of the

lysozyme gene in the cow (Irwin *et al.*, 1989) but only one in the human (Peters *et al.*, 1989).

Milk protein cDNA and genomic sequences

The available cDNA sequences for the various milk proteins are listed in *Table 1* and *Table 2*, while available gene sequences are listed in *Table 3*.

Table 1. The currently available casein cDNAs. Since all the casein genes sequenced so far can be classified as homologues of the four archetypal bovine caseins, their cDNA sequences are presented accordingly

Casein type	Species	Reference
α_{S1}-like Caseins	Cow	Stewart, Willis and Mackinlay (1984); Nagao *et al.* (1984)
	Rat α	Hobbs and Rosen (1982)
	Guinea-pig B	Hall, Laird and Craig (1984)
	Sheep	Mercier *et al.* (1985)
	Rabbit	Devinoy *et al.* (1988)
	Goat	Brignon *et al.* (1990)
	Pig	Alexander and Beattie (1992a)
	Tammar wallaby	Collet, Joseph and Nicholas (1992)
α_{S2}-like Caseins	Cow	Stewart *et al.* (1987)
	Mouse ε	Hennighausen, Steudle and Sipple (1982)
	Rat γ	Hobbs and Rosen (1982)
	Guinea-pig A	Hall *et al.* (1984)
	Sheep	Boisnard and Petrissant (1985)
	Pig	Alexander, Das Gupta and Beattie (1992)
	Rabbit	Dawson *et al.* (1993)
β-Caseins	Cow	Stewart *et al.* (1987)
	Rat	Blackburn, Hobbs and Rosen (1982)
	Mouse	Yoshimura, Banerjee and Oka (1986)
	Rabbit	Schaerer *et al.* (1988)
	Sheep	Provot, Persuy and Mercier (1989)
	Pig	Alexander and Beattie (1992b)
	Human	Lonnerdal *et al.* (1990)
	Tammar wallaby	Collet, Joseph and Nicholas (1992)
κ-Caseins	Cow	Stewart, Willis and Mackinlay (1984)
	Rat	Nakhasi, Grantham and Gullino (1984)
	Mouse	Thompson, Dave and Nakhasi (1985)
	Pig	Levine *et al.* (1992)
	Guinea-pig	Hall (1990)
	Sheep	Furet *et al.* (1990)
	Rabbit	Bosze *et al.* (1993)

Chromosomal locations

The four bovine casein genes have been mapped to chromosome 6 at q31–33 (Womack *et al.*, 1989). From inheritance studies of the bovine caseins it was initially suggested that their genes occur as a cluster in the order α_{S1}–α_{S2}–β–κ (Grosclaude, Joudrier and Mahe, 1979). More recently, long-range restriction mapping has shown them to be in the order α_{S1}–β–α_{S2}–κ within a 200 kb region, although there is disagreement as to the distances separating them (Ferretti, Leone and Sgaramella, 1990; Threadgill and Womack, 1990).

Table 2. The currently available whey protein cDNAs for some milk protein genes

Protein type	Species	Reference
α-Lactalbumin	Human Guinea-pig	Hall *et al.* (1992)
	Rat	Dandekar and Qasba (1981)
	Sheep	Gaye *et al.* (1987)
	Goat	Kumagai *et al.* (1987)
	Tammar wallaby	Collet, Joseph and Nicholas (1990)
	Mouse	Vilotte, Soulier and Mercier (1992)
	Pig	Das Gupta, Alexander and Beattie (1992)
β-Lactoglobulin	Cow	Alexander *et al.* (1989)
	Sheep	Gaye *et al.* (1986)
	Pig	Alexander and Beattie (1992c)
	Tammar wallaby	Collet, Joseph and Nicholas (1991)
Whey acidic protein	Rat and mouse	Hennighausen *et al.* (1982)
Late lactation protein	Tammar wallaby	Collet, Joseph and Nicholas (1989)
Lactoferrin	Mouse	Rey *et al.* (1990)
Lysozyme	Cow	Steinhoff, Senft and Seyfert (1994)

Table 3. The currently available sequences for milk protein genes

Protein type	Species	Reference
α_{s1}-like Caseins	Cow	Koczan, Hobom and Seyfert (1991)
	Goat	Leroux, Mazure and Martin (1992)
	Rabbit	Jolivet *et al.* (1992)
	Rat 5' region	Yu-Lee *et al.* (1986)
α_{s2}-like Caseins	Cow	Groenen *et al.* (1993)
	Rat 5' region	Yu-Lee *et al.* (1986)
β-Casein	Cow	Bonsing *et al.* (1988); Gorodetskii, Tkach and Kapelinskaya (1988)
	Mouse	Yoshimura and Oka (1989)
	Rat	Jones *et al.* (1985)
	Goat	Roberts *et al.* (1992)
	Rabbit	Thepot *et al.* (1991)
	Human	Hansson *et al.* (1994a)
κ-Casein	Cow	Alexander *et al.* (1988)
α-Lactalbumin	Cow	Vilotte *et al.* (1987)
	Rat	Qasba and Safaya (1984)
	Human	Hall *et al.* (1987)
	Guinea-pig	Laird *et al.* (1988)
	Goat	Vilotte *et al.* (1991)
	Mouse	Vilotte and Soulier (1992)
β-Lactoglobulin	Cow	Alexander *et al.* (1993)
	Sheep	Ali and Clarke (1988)
Whey acidic protein	Rat and mouse	Campbell *et al.* (1984)
	Rabbit	Thepot *et al.* (1990)
Lactoferrin	Mouse partial	Cunningham, Headon and Conneely (1992)
	Human 5' region	Teng *et al.* (1992)
Lysozyme	Human	Peters *et al.* (1989)

The human β-casein gene has been mapped to position 4pter → q21 (Menon *et al.*, 1992). The casein genes have been mapped to chromosome 5 in the mouse (Gupta *et al.*, 1982), to position q32 of chromosome 4 in the sheep (Hayes *et al.*, 1992), and to

position q24 of chromosome 12 in the rabbit (Gellin *et al.*, 1985). Hayes *et al.* (1992) also suggested that the assignment of the bovine caseins to chromosome 6 may be an error caused by the very similar banding patterns of chromosomes 4 and 6. However, according to Toldo *et al.* (1994), this confusion is caused by differences in the Reading and ISCNDA (International System for Cytogenetic Nomenclature of Domestic Animals) standards, where the ISCNDA chromosome 4 is actually the Reading chromosome 6. They therefore recommend that the bovine caseins be assigned to the Reading chromosome 6 and that the ISCNDA designation be changed accordingly.

The α-lactalbumin gene has been mapped to chromosome 5 in the cow and goat, to chromosome 3 in the sheep (Hayes, Popescu and Dutrillaux, 1993), and to position q13 of chromosome 12 in the human (Davies *et al.*, 1987).

The BLG gene has recently been mapped to position q28 on chromosome 11 of the cow, and to chromosomes 11 and 3 of the goat and sheep, respectively (Hayes and Petit, 1993).

The late lactation protein has been mapped to the proximal region of the long arm of chromosome 3 in the tammar wallaby (Westerman, Spencer and Collet, 1991). The lactoferrin gene has been mapped to chromosome 9 in the mouse and to chromosome 3 in the human (Teng *et al.*, 1987), while the human lysozyme gene locus has been mapped to chromosome 12 (Peters *et al.*, 1989).

Evolution and gene structure

THE CALCIUM-SENSITIVE CASEINS

It has been proposed that the α_{S1}- α_{S2}- and β-caseins evolved from a common ancestor which appeared about 220–400 million years ago (Hobbs and Rosen, 1982), by a series of intra- and intergenic duplications and exon shuffling events (Bonsing and Mackinlay, 1987). Alignments of the casein cDNAs from several different species show that there is little sequence conservation between different caseins within a species. Such conservation is confined to the sequences encoding the 5' untranslated regions, leader peptides and casein kinase phosphorylation sites of the calcium-sensitive caseins. The high degree of conservation of the leader peptide may be a consequence of it either being unusually short and so having a more limited capacity for variation, or of its nucleotide sequence being involved in gene expression. The lack of conservation of the majority of the coding region is consistent with the perceived loose structure–function relationship of the caseins in forming stable micelles (Bonsing and Mackinlay, 1987).

It has been proposed that the ancestral gene from which the caseins are derived would have required four essential elements: (1) the 5' non-coding region, (2) the leader peptide, (3) a hydrophilic sequence containing a major phosphorylation site, and (4) a hydrophobic sequence (Jones *et al.*, 1985). It is most probable that while the ancestral gene would retain these elements, duplicated copies of it would be free to evolve positively selectable functions.

Sequence comparisons of the β-casein genes from different species show that there are no major insertions, deletions or rearrangements, and substitutions tend to be conservative. Sequence comparisons of the α-like caseins show that they have

undergone many insertion/deletion events. These comparisons imply that the β-casein gene is most closely related to the ancestral gene, while the α-like caseins have been free to diverge from this ancestral sequence. The divergence of the α_{S1}- and then α_{S2}-casein genes may have been in response to selective pressure for higher levels of calcium phosphate in the milk, necessary for faster growth of the infant (Bonsing and Mackinlay, 1987).

It seems that the α_{S1}-casein gene is the product of the first duplication event as the 5' non-coding regions of the β- and α_{S2}-like caseins are more similar than those of the β- and α_{S1}- like caseins (Bonsing and Mackinlay, 1987). This proposal is in agreement with the work of Groenen *et al.* (1993) who, from similarities between the first three exons of the α_{S2}- and β-casein genes, and also between the α_{S2}-casein exon 14 and the β-casein exon 5, suggested that not only are these two genes most closely related, but that they could have arisen from a common ancestral gene by as little as two duplication events. Interestingly, two α_{S2}-casein cDNAs which are sufficiently different to indicate the presence of two active α_{S2}-casein genes, have recently been isolated from the rabbit. Presumably these arose from a gene duplication event which, from their degree of sequence similarity, appears to have occurred relatively recently (Dawson *et al.*, 1993).

The genes encoding the α_{S1}- and α_{S2}-caseins are large and have many small exons, some of which are thought to be the result of internal duplications. This is especially apparent in the bovine α_{S1}-casein gene, where six exons all conform to the criterion GAX–six codons–XAG (Stewart, Willis and Mackinlay, 1984; Koczan, Hobom and Seyfert, 1991). Two of the exons (10 and 13) display 23/24 identity and are contained within duplicated 154 bp regions which may be the product of a gene conversion event. In the α_{S2}-casein gene it is apparent that exons 7–11 and 12–16 are the product of an internal duplication event (Stewart *et al.*, 1987), while the region containing exon 3 displays 75% identity to the region containing exon 12, and so may be a consequence of a similar event (Groenen *et al.*, 1993).

The organization of all three calcium-sensitive casein-encoding genes retains an arrangement whereby the first intron is near the end of the 5' untranslated region, with the second exon containing the remainder of the 5' untranslated region, the leader peptide and the first two amino acids of the mature protein. At the other end of these genes, the second to last exon is short and contributes either some or all of the stop codon, with the final exon contributing the remaining portion of the 3' untranslated region.

κ-CASEIN

The κ-casein gene consists of several small exons and a single large exon that encodes most of the mature protein. Of the various caseins, κ-casein displays the greatest conservation between species (Nakhasi *et al.*, 1984; Thompson, Dave and Nakhasi, 1985), which is in keeping with its structural role in the stabilization of casein micelles. There is no significant similarity to any of the α_{S1}-, α_{S2}- or β-casein genes, which has led to the proposal that the κ-casein gene was recruited into the casein cluster from an ancestral gene unrelated to the other caseins, possibly γ-fibrinogen (Alexander *et al.*, 1988). This proposal may need revision in the light of the suggestion by Weissbach and Greininger (1990) that the fibrinogens themselves are derived from two ancestral genes.

α-LACTALBUMIN

α-Lactalbumin is a calcium metalloprotein which associates with the enzyme galactosyltransferase in the Golgi bodies to form the lactose synthetase complex (Ebner and Schanbacher, 1974). It is thought that the α-lactalbumin gene and the members of the lysozyme gene family share a common ancestor. Not only do the cDNA sequences of human α-lactalbumin and chicken lysozyme have significant identity (Hall *et al.*, 1982), but the rat (Qasba and Safaya, 1984), human (Hall *et al.*, 1987), bovine (Vilotte *et al.*, 1987) and guinea-pig (Laird *et al.*, 1988) α-lactalbumin, and the chicken (Jung *et al.*, 1980) and human (Peters *et al.*, 1989) lysozyme genes contain three introns at similar positions, and the first three of the four exons are comparable in both sequence and size. In the mouse (Vilotte and Soulier, 1992), and the rat, human and guinea-pig (Soulier *et al.*, 1989), the α-lactalbumin gene has been reported to occur as a single copy. In both the cow and the sheep more than one α-lactalbumin-like gene has been reported (Soulier *et al.*, 1989). In each species an α-lactalbumin pseudogene was discovered which at its 3' end shares identity with the 3' half of the α-lactalbumin gene. The 5' end of these pseudogenes share identity only with each other and it appears that they arose before the two species themselves diverged (Soulier *et al.*, 1989). A second bovine α-lactalbumin pseudogene with identity to the 3' half of the α-lactalbumin gene has recently been reported. It is flanked by repeated LINE sequences which have also been found associated with the lysozyme gene family, and which may be involved in the evolution of these genes (Vilotte, Soulier and Mercier, 1993).

β-LACTOGLOBULIN

In most species only one active BLG gene is known to exist. However, horse (Conti *et al.*, 1984), dog and dolphin (Pervaiz and Brew, 1986), and donkey (Godovac-Zimmerman *et al.*, 1988) are thought to have two distinct active genes, while the cat appears to have three (Halliday *et al.*, 1993). In these latter cases the BLG proteins are monomeric and, with the exception of the cat, are termed BLGI and BLG II for the major and minor components, respectively. In the cat, BLG I, II and III are all deemed to be major components. Recently, a BLG pseudogene has been isolated from the cow which, from the predicted amino acid sequence of the ancestral translation product, displays greater identity to the BLG IIs than to bovine BLG. It may therefore be derived from a BLG II gene in the cow (Passey and Mackinlay, in preparation).

The BLG protein is structurally similar to the members of a genetically related protein family known as the lipocalins. This diverse family is predominantly composed of low molecular weight secretory proteins that have a strong and specific affinity for hydrophobic molecules (reviewed by Flower, North and Attwood, 1993). A conserved folding pattern and three-dimensional structure, rather than strong sequence similarity, are the defining characteristics for members of this family. Despite the lack of sequence identity, the gene structure of a number of the lipocalins has been shown to be similar to that of the BLG gene, suggesting that the members of this family are derived from a common ancestral gene (Ali and Clark, 1988). The lipocalin gene which is most similar to the BLGs, in terms of size, exon arrangement and sequence identity, is the human

PP14 gene (Vaisse *et al.*, 1990) which encodes a 43 kDa glycoprotein secreted in the endometrium during pregnancy (Bell *et al.*, 1985).

WHEY ACIDIC PROTEIN

No other genes related to the rat, mouse or rabbit whey acidic protein genes have been reported either in these or other species. The rat and mouse genes extend over 2.8 kb and 3.3 kb, respectively, and are both composed of four exons. The third and fourth exons each encode a cysteine domain and are thought to have arisen by intragenic duplication of a primordial exon (Hennighausen *et al.*, 1982).

Comparison of the rat and mouse cDNA sequences shows a greater degree of conservation between the 3' non-coding regions than between the coding regions (Hennighausen *et al.*, 1982). Comparison of the rat and mouse genes shows that the introns are only slightly less conserved than the exons (Campbell *et al.*, 1984). These observations not only indicate a loose structure–function relationship for the mature protein, but also suggest a role for the 3' untranslated region in the production and maintenance of a stable transcript.

LATE LACTATION PROTEIN

Although no function of the late lactation protein (LLP) is known for certain (apart from acting as a source of amino acids), its similarity to BLG has prompted the suggestion that it may transport some small ligand released during the hydrolysis of milk fat (Collet and Joseph, 1993). The late lactation protein is found in the tammar wallaby (*Macropus eugenii*), and also possibly in the red (*Macropus rufus*) and eastern grey *Macropus giganteus*) kangaroos, and in the quokka (*Setonix brachyurus*) (Nicholas *et al.*, 1987, and references therein). No LLP gene has yet been sequenced.

It has recently been suggested that the LLP is a member of the lipocalin superfamily. A search of the GenPeptide and NBRF-PIR protein databases revealed homologies to a number of the members of this family, including BLG (Collet and Joseph, 1993). Late lactation protein and BLG share 42% and 20% similarity at the cDNA and protein levels, respectively, and it has been suggested that they share a common ancestor from which they diverged 150 million years ago (Woodlee *et al.*, 1993). The members of the lipocalin family to which LLP has the greatest similarity are the non-core members – rat and human von Ebner's gland protein and rat odorant-binding protein – implying that LLP is also a non-core member (Collet and Joseph, 1993).

LACTOFERRIN

Comparison of the amino acid sequences of the lactoferrin, serum transferrin, ovotransferrin and melanotransferrin proteins showed that, in addition to their overall homology, the N-terminal and C-terminal halves are approximately 40% identical to each other (Metz-Boutigue *et al.*, 1984; Bowman, Yang and Gwendolyn, 1988). The similarity of these proteins has since been confirmed at the level of gene structure in that they have a similar exon–intron distribution pattern (Cunningham, Headon and Conneely, 1992). It has therefore been proposed that they form a gene family which originated approximately 200–500 million years ago from a common ancestral gene

which was itself a product of an internal duplication event. This event was apparently favoured by selective pressure as it resulted in the duplication of an iron-binding site in the protein (Williams *et al.*, 1982).

Milk protein transgenics

The characterization and cloning of the milk protein genes, together with the advent of transgenic technology, presented the potential to express biologically active proteins in the milk of transgenic animals (Palmiter *et al.*, 1982; Lovell-Badge, 1985). One advantage of harvesting foreign gene products from the body fluids of transgenic animals is that they are a renewable source, while harvesting recombinant proteins from tissue would almost certainly necessitate slaughtering valuable animals (Clark *et al.*, 1987). By targeting gene expression to the liver, for example, foreign proteins have been secreted into the blood of transgenic animals (reviewed by Ebert and Schindler, 1993). A possible problem with this approach is that biologically active foreign proteins circulating in the bloodstream could have an adverse effect on the health of the animal. Another restriction is the volume of blood able to be harvested. Recombinant proteins have been successfully targeted via the liver to the serum of pigs and sheep, but so far expression levels have been no greater than 1 $\mu g\ ml^{-1}$ for proteins other than serum proteins (Ebert and Schindler, 1993). In contrast, the mammary gland of mammals is able to synthesize and secrete large amounts of foreign protein during each lactation. Sheep, for example, are able to produce approximately 100 g of protein/day, while cows can produce up to 1 kg/day (Clark *et al.*, 1989a). An additional advantage of utilizing the mammary gland as a bioreactor is that the technology to harvest large quantitites of milk is already well established and non-invasive, thereby reducing stress on the animal (Logan, 1993).

Unlike bacterial expression systems, (see below), a number of transgenic experiments have demonstrated that the mammary gland is able to perform a variety of post-translational modifications on heterologous protein products. These include β-hydroxylation (Clark *et al.*, 1989b), glycosylation (Ebert *et al.*, 1991; Wright *et al.*, 1991) and vitamin K-dependent γ-carboxylation (Velander *et al.*, 1992). In addition to performing these post-translational modifications, the mammary gland is also able to synthesize cysteine-rich foreign proteins which are able to fold into a functional conformation (Meade *et al.*, 1990), as well as heterodimeric protein complexes (Greenberg *et al.*, 1991), transmembrane proteins (DiTullio *et al.*, 1992) and protease-sensitive metalloproteins (Hansson *et al.*, 1994b).

To date, a number of high-value biologically active human proteins have been synthesized and secreted by the mammary tissue of mice, rats, rabbits, pigs, sheep and goats. These include such complex proteins as blood clotting factor IX (FIX; Clark *et al.*, 1989b), tissue plasminogen activator (t-PA; Gordon *et al.*, 1987; Ebert *et al.*, 1991), α_1-antitrypsin (α_1AT; Simons *et al.*, 1988; Archibald *et al.*, 1990; Wright *et al.*, 1991; Bischoff *et al.*, 1992), interleukin-2 (hIL-2; Bühler *et al.*, 1990), urokinase (Meade *et al.*, 1990), growth hormone (Reddy *et al.*, 1991; Ninomiya *et al.*, 1994), protein C (Velander *et al.*, 1991, 1992), cystic fibrosis transmembrane conductance regulator (hCFTR; DiTullio *et al.*, 1992), serum albumin (hSA; Shani *et al.*, 1992), extracellular superoxide dismutase (Hansson *et al.*, 1994b), lactoferrin (Platenburg *et al.*, 1994) and lysozyme (Maga *et al.*, 1994).

Before reviewing the progress of milk transgenics in detail, it will be useful to consider briefly the two main alternatives for the production of pharmaceutical proteins, namely microbial fermentation and mammalian cell culture.

Microbial fermentation and mammalian cell culture

Bacterial fermentation has been the system of choice for the production of relatively small and unmodified recombinant proteins, such as insulin, and hIL-2 (Reisman, 1993). However, many human proteins require post-translational modifications such as phosphorylation, glycosylation, amidation and proteolytic cleavage in order to acquire or maintain biological activity. Generally, bacterial systems do not possess such post-translational machinery and are therefore unable to synthesize these complex compounds (Bebbington and Hentschel, 1985). An additional problem is the relatively high reducing environment within bacteria, which can result in failure to form the correct disulphide bonds and therefore the formation of a non-functional protein product (Bebbington and Hentschel, 1985). Many mammalian proteins expressed within *Escherichia coli*, for example, fail to fold correctly and are deposited within inclusion bodies (Marston, 1986). For example, some proteins, such as t-PA and prochymosin, are completely insoluble within *E. coli* (Yarranton, 1990). Considerable downstream processing is necessary to solubilize and refold these proteins to restore biological activity. This process may dramatically reduce product yield and is very expensive to perform on an industrial scale (Bebbington and Hentschel, 1985; Datar, Cartwright and Rosen, 1993).

Although yeasts possess the ability to glycosylate heterologous proteins, they do so with very low efficiency (Wood *et al.*, 1985; Cabezón *et al.*, 1984). For example, human α_1AT has been expressed in *E. coli* and yeast, however the recombinant proteins are not glycosylated, drastically reducing their serum half-life (Casolaro *et al.*, 1987). The recombinant protein produced by lactating transgenic sheep, however, seems to be identical to that isolated from human plasma (Wright *et al.*, 1991). Aberrant glycosylation has also been documented in yeast. Such is the case for human insulin-like growth factor-I (IGF-I) synthesized by the yeast *Saccharomyces cerevisiae*, some of which was found to be *O*-glycosylated in contrast to the natural human product (Gellerfors *et al.*, 1989). This hyperglycosylation also raises concerns of allergenicity and reduced serum half-life (Tekamp-Olson and Valenzuela, 1990; Hodgson, 1993).

An alternate route for pharmaceutical protein production is large-scale mammalian cell culture. Such is the case for recombinant t-PA which is synthesized by transformed Chinese hamster ovary (CHO) cells and marketed under the name of Activase by Genentech. However, large-scale mammalian cell culture is a very expensive process. A large proportion of this expense results from the high cost of culture media, especially fetal calf serum, and, in some cases, the low concentration of product secreted into the media, which results in greater downstream processing costs (Werner *et al.*, 1992; Datar, Cartwright and Rosen, 1993). Mammalian cell culture also suffers from technical difficulties due to slow doubling time, the low shear resistance of mammalian cells and problems with microbial contamination (Reisman, 1993).

Mammary gland-specific transgenic studies

This section provides a brief description of the approaches that are used in transgenesis, i.e. the generation and study of transgenic animals. It then proceeds to summarize work done using mammary-specific promoters, derived from milk protein genes, to direct expression of both milk proteins and heterologous proteins in the mammary gland.

TRANSGENESIS

A transgenic organism is defined as one containing a foreign gene integrated into its own genome (Smith, Meuwissen and Gibson, 1987). Methods of introducing exogenous DNA into the germline include pronuclear microinjection, homologous recombination in embryonic stem (ES) cells and the use of retroviral vectors (reviewed by Thompson *et al.*, 1993). Recently, methods have been developed to transform various somatic tissues of mature animals via jet injection (Furth *et al.*, 1992) and particle bombardment (Klein and Fitzpatrick-McElligott, 1993). These techniques have been termed 'somatic transgenesis' as they transform existing tissues. These methods have already proven useful in assessing transgenic constructs without the need to first introduce them into the germline (Furth *et al.*, 1992; Cheng, Ziegelhoffer and Yang, 1993).

The most common method of introducing exogenous DNA into the germline of animals is via microinjection into the pronucleus of a single-cell embryo (Gordon *et al.*, 1980). This process involves piercing the pronucleus with an extremely fine needle and directly injecting a DNA solution (approximately 2 pl), containing the transgene construct, at a concentration of 1–2 $\mu g\ ml^{-1}$. The pronuclei of mice, sheep, goats and rabbits are easily resolved and targeted using differential interference contrast microscopy; however, the opacity of porcine and bovine eggs necessitates centrifugation for visualization. This process stratifies the cytoplasm, resulting in the deposition of the pronuclei in a transparent equatorial layer of the egg, allowing them to be resolved and injected (Hammer *et al.*, 1985; Wall *et al.*, 1985). There appears to be no limit to the size of DNA molecules that can be microinjected and integrated into the host genome. Recently, fragments as large as 230 kb have been introduced into mice (Schedl *et al.*, 1993). After microinjection, surviving embryos are reimplanted into pseudopregnant foster mothers where normal development may continue. After birth, transgenic individuals may be identified using Southern hybridization with DNA probes specific for the transgene or, alternatively, by the polymerase chain reaction (PCR; Saiki *et al.*, 1988).

In the case of mice, approximately 25% of the pups that are born will contain one or more copies of the transgene (Brinster *et al.*, 1985). Usually, multiple copies of the transgene integrate randomly at a single site within the genome, therefore each transgenic individual produced via microinjection is unique (Smith, Meuwissen and Gibson, 1987; Pursel *et al.*, 1989). About 10–20% of transgenic individuals contain the transgene integrated at multiple chromosomal locations (Brinster *et al.*, 1985). When multiple copies of the injected DNA integrate into the genome at a single site they are usually arranged in a tandem head-to-tail array (Brinster *et al.*, 1981; Palmiter *et al.*, 1982). Generally, provided it is present in the germline, the integrated DNA is

stably transmitted from generation to generation. The precise mechanism by which the microinjected DNA integrates into the genome remains unknown (Palmiter and Brinster, 1986).

At present, pronuclear microinjection is the only method available to alter the germline of higher mammals, and has been used successfully to transform mice, rabbits, goats, pigs, sheep and cows (Gordon *et al.*, 1980; Hammer *et al.*, 1985; Ebert *et al.*, 1991; Hill *et al.*, 1992). The use of homologous recombination in pluripotent ES cells allows better control over the site of transgene integration and copy number, as well as the ability to remove a specific gene and replace it with another (Capecchi, 1989; Valancius and Smithies, 1991; Stacey *et al.*, 1994). However, at present this technology exists only for mice.

TRANSGENIC EXPRESSION

Transgenic studies have demonstrated that genes from one mammalian species are able to be expressed within another. In addition, genes normally expressed in one particular tissue, such as milk protein genes, usually maintain tissue-specific gene expression when introduced into another mammalian species (Brinster *et al.*, 1983; Storb *et al.*, 1984; Swift *et al.*, 1984; Palmiter and Brinster, 1986; Simons, McClenaghan and Clark, 1987).

Typically, the expression of a particular transgene varies among the different transgenic lines and exhibits no correspondence between expression levels and transgene copy number (Lee *et al.*, 1988; Bonnerot *et al.*, 1990). It is thought that this is caused by the transcriptional state of the surrounding host chromatin influencing transgene expression levels (Allen, Norris and Surani, 1990; Al-Shawi *et al.*, 1990).

Recently, genetic elements, termed dominant control regions (DCRs), have been identified upstream of the human β-globin locus. These elements confer position-independent, copy number-dependent expression of both the cognate gene as well as those under the control of heterologous promoters (Grosveld *et al.*, 1987; van Assendelft *et al.*, 1989). Similar elements have been found downstream of the human CD2 gene (Greaves *et al.*, 1989). These elements are thought to delineate active chromatin regions and are thus able to insulate transgenes from the environmental effects stemming from the surrounding host chromatin. As a consequence, the level of transgene expression becomes proportional to the number of gene copies integrated (Patient, 1990; Felsenfeld, 1992).

TRANSGENIC STUDIES UTILIZING MAMMARY-SPECIFIC PROMOTERS

At present promoter elements from ovine BLG (oBLG; Archibald *et al.*, 1990; Shani *et al.*, 1992), murine WAP (Gordon *et al.*, 1987; Pittius *et al.*, 1988; Yu *et al.*, 1989; Reddy *et al.*, 1991; Hansson *et al.*, 1994b), rabbit WAP (Bischoff *et al.*, 1992), bovine α-lactalbumin (Stinnakre *et al.*, 1991), rat β-casein (Greenberg *et al.*, 1991), goat β-casein (DiTullio *et al.*, 1992) and bovine α_{S1}-casein (Meade *et al.*, 1990; Hyttinen *et al.*, 1994; Maga *et al.*, 1994; Platenburg *et al.*, 1994) have been utilized to express foreign proteins in the mammary gland of transgenic mice. Endogenous promoters have been utilized in sheep (Simons *et al.*, 1988; Clark *et al.*, 1989b; Wright *et al.*, 1991), rabbits (Bühler *et al.*, 1990) and goats (Ebert *et al.*, 1991), while murine WAP

Table 4. Mammary-specific transgene expression studies in mice; listed according to each mammary-specific promoter

Transgene construct	Flanking sequence[†]	Expression level/ml[§]	Tissue specificity[¶]	Reference
β-lactoglobulin (BLG)				
oBLG	4.0; 7.3 kb	3–23 mg	MG specific	Simons, McClenaghan and Clark (1987)
oBLG–hα_1AT	4.0 kb; α_1AT	0.5–7 mg	MG, SG	Archibald *et al.* (1990)
oBLG–hSA (minigene)	3.0 kb	1–10 mg	Not reported	M. Shani (personal communication)
Whey acidic protein (WAP)				
mWAP–ht-PA (cDNA)	2.6 kb; SV40	<20 ng–50 μg	MG, T, K, SuG	Gordon *et al.* (1987)
mWAP–hCD4 (cDNA)	1.85 kb; bGH	10–200 ng	Not reported	Yu *et al.* (1989)
mWAP	2.6; 1.6 kb	3–54% endog[‡]	Not reported	Burdon *et al.* (1991)
mWAP–hGH	2.6 kb; hGH	65 ng–410 μg; 3.5 mg	MG, serum	Reddy *et al.* (1991)
rWAP	949; 70 bp	0–500% endog[‡]	Not reported	Dale *et al.* (1992)
RWAP–h α_1AT	17.6 kb; α_1AT	6–10.5 mg	Not reported	Bischoff *et al.* (1992)
mWAP-hSOD (cDNA)	2.3; 4 kb	8 ng–700 μg	MG, brain	Hansson *et al.* (1994b)
α-lactalbumin				
b α-lactalbumin	750; 336 bp	0.0025–0.45 mg	MG specific	Vilotte *et al.* (1989)
b α-lactalbumin–oTP (cDNA)	750; 450 bp	1 μg	MG specific	Stinnakre *et al.* (1991)
gp α-lactalbumin	1195; 398 bp	Not reported	Leaky	Maschio *et al.* (1991)
g α-lactalbumin	8.5; 9.5 kb	1.2–3.7 mg	Leaky	Soulier *et al.* (1992)
β-casein				
r β-casein	3.5; 3 kb	0.01–1% endog[‡]	MG, brain	Lee *et al.* (1988)
r β-casein–bFSHα (cDNA)	524 bp; SV40	15 μg[f]	Not reported	Greenberg *et al.* (1991)
r β-casein–bFSHβ (cDNA)	524 bp; SV40		Not reported	Greenberg *et al.* (1991)
g β-casein (18 kb)	3.0; 6.0 kb	21–24 mg; 40 mg	MG, skin	Persuy *et al.* (1992)
g β-casein (18.5 kb)	4.2; 5.3 kb	1 mg	MG, skin	Roberts *et al.* (1992)
g β-casein–hCFTR (cDNA)	4.2; 5.3 kb	Not reported	MG specific	DiTullio *et al.* (1992)
α_{S1}-casein				
b α_{S1}-casein–h urokinase	21; 2 kb	1–2 mg	MG specific	Meade *et al.* (1990)
b α_{S1}-casein–hLF (cDNA)	6.2; 8 kb	0.1–36 μg	MG specific	Platenburg *et al.* (1994)
b α_{S1}-casein-hLZ (cDNA)	20; 2 kb	to 0.78 mg	MG specific	Maga *et al.* (1994)

[†] Flanking sequence around the designated transcriptional unit (size of 5' flanking; 3' flanking sequence).
[§] The range of transgene expression levels in milk. First set of values gives range of founder expression levels, i.e. hemizygote expression. Values after; give highest homozygote expression level if reported.
[‡] mRNA expression levels determined only.
[f] Bigenic expression level.
[¶] Transgene expression highly mammary gland (MG) specific or leaky expression into other tissues.
Abbreviations: α_1AT, α_1-antitrypsin; b, bovine; CFTR, cystic fibrosis transmembrane conductance regulator; FSH, follicle-stimulating hormone; g, goat; GH, growth hormone; gp, guinea-pig; h, human; K, kidney; LF, lactoferrin; LZ, lysozyme; m, mouse; MG, mammary gland; o, ovine; r, rat; R, rabbit; SA, serum albumin; SG, salivary gland; SOD, extracellular superoxide dismutase; SuG, sublingual gland; T, tongue; TP, trophoblast interferon; t-PA, tissue plasminogen activator.

has been utilized successfully in pigs (Ebert *et al.*, 1991; Velander *et al.*, 1992). Transgenic cattle have also been generated (Krimpenfort *et al.*, 1991; Hill *et al.*, 1992; Hyttinen *et al.*, 1994).

We will now discuss many of the transgenic experiments published using various mammary-specific promoters. Summaries of these studies in mice and other mammals are presented in *Table 4* and *Table 5*, respectively.

β-*Lactoglobulin*

β-Lactoglobulin (BLG) is the major whey protein of ruminants and is present at a concentration of 4.6 mg ml^{-1} in the milk of cows (Caffin, Poutrel and Rainard, 1985). Ovine β-lactoglobulin (oBLG) became the first transgenic milk protein to be expressed in another organism when Simons, McClenaghan and Clark (1987) introduced the genomic sequence into mice. The transgene contained 4 kb of 5' sequence, the entire oBLG transcriptional unit and 7.3 kb of 3' flanking sequence. Lactating transgenic mice were found to produce a new protein in their milk, the identity of which was confirmed as oBLG by Western blotting. Expression levels between different transgenic mice varied from 3 to 23 mg ml^{-1}, the latter being more than four times the endogenous expression level in sheep. The presence of the foreign gene product did not appear to affect the lactating mothers or the suckling pups adversely. Analysis of RNA isolated from a variety of tissues detected oBLG mRNA specifically in the lactating mammary gland, thus the oBLG gene was expressed in a tissue- and stage-specific manner. Whitelaw *et al.* (1992) have subsequently demonstrated that only the first 406 bp of 5' flanking sequence and 1.9 kb of 3' sequence are necessary to confer position-independent, copy-number-related expression of the oBLG transgene.

The ability of the oBLG promoter to drive the expression of foreign genes in sheep was examined with constructs containing both the cDNA sequence of α_1AT and FIX (Simons *et al.*, 1988). FIX is used in replacement therapy to treat sufferers of haemophilia B, or Christmas disease, a genetic deficiency of blood clotting factor IX which affects approximately 1 in 30 000 males (Brownlee, 1987). FIX is usually isolated from human serum; however, supply is limited and the blood-derived product has the added risk of viral contamination. Active protein cannot be produced by bacterial systems as it requires extensive post-translational modifications for activity. Active α_1AT, on the other hand, can be synthesized by micro-organisms, but due to the lack of carbohydrate side-chains the protein has a greatly reduced serum half-life (Garver *et al.*, 1987).

The oBLG–FIX construct contained the FIX cDNA sequence cloned into the *Pvu* II site in the 5' untranslated portion of the first exon of the oBLG gene and possessed 4.3 kb of upstream oBLG sequence (Simons *et al.*, 1988). FIX cDNA was used as the genomic sequence of human FIX is 33.5 kb in length and therefore difficult to manipulate (Yoshitake *et al.*, 1985). Lactating ewes, containing the above, construct expressed biologically active FIX in their milk; however, the expression level was very low. Maximal expression was just 25 ng ml^{-1}, 1/250th the concentration in human plasma and 1/100 000th the concentration of oBLG in milk (Clark *et al.*, 1989b). The maximal expression level obtained with the oBLG-α_1AT construct was 5 μg ml^{-1}, which is higher than the oBLG–FIX construct, but is still 1000 times lower than endogenous oBLG expression in sheep. As will be discussed below, there tends

Table 5. Mammary-specific transgene expression studies in rats, sheep, rabbits, goats, pigs and cattle, arranged according to species

Transgene construct	Flanking sequence[†]	Expression level/ml[§]	Tissue specificity[¶]	Reference
Rat				
b α-lactalbumin	0.8; 0.5 kb	0.2 μg–2.4 mg	Not reported	Hochi *et al.* (1992)
b α-lactalbumin–hGH	738 bp; hGH	1 μg–4.4 mg	MG, serum	Ninomiya *et al.* (1994)
b α_{s1}-casein–hGH	671 bp; hGH	87 μg–6.5 mg	MG, serum	Ninomiya *et al.* (1994)
b β-casein–hGH	1.7 kb; hGH	1 μg–10.9 mg	MG, serum	Ninomiya *et al.* (1994)
m WAP–hGH	2.6 kb; hGH	7–56 μg	MG, serum	Ninomiya *et al.* (1994)
Sheep				
oBLG–h α_1AT (cDNA)	4.9; 7.3 kb	5 μg	Not reported	Simons *et al.* (1988)
oBLG–h Factor IX (cDNA)	4.9; 7.3 kb	25 ng	Not reported	Clark *et al.* (1989b)
oBLG–h α_1AT	4.0 kb; α_1AT	1–35, 63 mg*	MG specific	Wright *et al.* (1991)
Rabbits				
R β-casein–h IL-2	2.0 kb; IL-2	50–430 ng	Not reported	Bühler *et al.* (1990)
Goats				
mWAP–ht-PA (cDNA)	2.6 kb; SV40	3–6 μg	Not reported	Ebert *et al.* (1991)
g β-casein–ht-PA (cDNA)	6.2; 7.1 kb	1–3 mg	MG specific	Ebert *et al.* (1994)
Pigs				
mWAP	2.6; 1.6 kb	1–2 mg	MG, SG	Wall *et al.* (1991)
mWAP–h protein C (cDNA)	2.6; 1.6 kb	0.001–1 mg	Not reported	Velander *et al.* (1992)
Cattle				
b α_{s1}-casein–hLF (cDNA)	15; 6 kb	Too early to assess		Krimpenfort *et al.* (1991)
b α_{s1}-casein–hEPO	1.5; 1.7 kb	Too early to assess		Hyttinen *et al.* (1994)
MMTV–hIGF-I	2.0 kb	Too early to assess		Hill *et al.* (1992)

[†] Flanking sequence around the designated transcriptional unit (size of 5' flanking; 3' flanking sequence).
[§] The transgene expression levels in milk.
[¶] Transgene expression highly mammary gland (MG) specific or leaky expression into other tissues.
* This sheep expressed the transgene at 63 mg ml^{-1} at the onset of lactation, then stabilized at 35 mg ml^{-1}.
Abbreviations: α_1AT, α_1-antitrypsin; b, bovine; EPO, erythropoietin; g, goat; GH, growth hormone; h, human;, IGF-I, insulin-like growth factor-I; IL-2, interleukin-2; LF, lactoferrin; m, mouse; MG, mammary gland; MMTV, murine mammary tumour virus; o, ovine; R, rabbit; SG, salivary gland; t-PA, tissue plasminogen activator.

to be a correlation between the use of cDNA based protein coding sequences and low transgene expression levels when compared to the use of whole gene sequences.

When the genomic sequence of α_1AT was linked to approximately 4.0 kb of the oBLG promoter, expression levels in transgenic mice increased dramatically to 0.5–7 mg ml^{-1} (Archibald *et al.*, 1990). The recombinant α_1AT was shown to be biologically active using a trypsin-inhibition assay. However, unexpectedly, some lines also expressed α_1AT in their salivary glands. This may be due to the absence from the construct of a salivary gland-specific negative regulatory element (Archibald *et al.*, 1990).

The genomic oBLG–α_1AT construct, described above, has also been introduced into sheep (Wright *et al.*, 1991). Two transgenic sheep produced α_1AT at levels of 1–5 mg ml^{-1}, while a third produced α_1AT at approximately 35 mg ml^{-1}, making the transgenic product the most abundant protein secreted into the milk. This same individual initially produced α_1AT at 63 mg ml^{-1} which then stabilized to about 35 mg ml^{-1}. Another sheep demonstrated the opposite effect, starting out at 0.9 mg ml^{-1}, then, at week 7, increased production to 3.5 mg ml^{-1}. The reasons for these variations are unknown. The identity of α_1AT present in the milk was confirmed by Western blotting, and it appeared to be completely *N*-glycosylated. These findings confirm the ability of the ovine mammary gland to perform *N*-glycosylation of foreign proteins and to secrete them in large quantities. The α_1AT was found to be bioactive and able to be isolated at greater than 95% purity by standard chromatographic procedures.

Shani *et al.* (1992) have performed extensive investigations into the requirements for the expression of foreign proteins in the milk of transgenic mice, using oBLG-based minigenes. Various constructs, comprising 3.0 kb oBLG and 0.8 kb of oBLG 5' and 3' flanking sequences respectively, hSA cDNA and hSA intron 1 or introns 1 and 2, were introduced into mice. A minigene containing the oBLG promoter, the first intron and the cDNA sequence of hSA expressed the encoded protein in the milk of transgenic mice at a level of 2.5 mg ml^{-1}. Transgenic mice possessing oBLG–hSA minigenes containing hSA introns 1+2+12–14 or 2+7–14 have been shown to express hSA at 1 mg ml^{-1} or greater (up to 10 mg ml^{-1}) in 20% of the mice generated (M. Shani, personal communication). These expression levels are higher than those reported using *E. coli* (Latta *et al.*, 1987) and yeast (Fleer *et al.*, 1991).

The bovine BLG gene has been cloned and sequenced in our laboratory (Alexander *et al.*, 1993). Recently, transgenic mice containing this gene have also been generated. All transgenic lines examined express the transgene, one in particular at a level several times that of the endogenous expression level in cow's milk (Bawden *et al.*, in preparation).

Whey acidic protein

Rat WAP has been expressed in transgenic mice (Bayna and Rosen, 1990; Dale *et al.*, 1992) while the murine gene has been expressed in both mice and pigs (Burdon *et al.*, 1991; Wall *et al.*, 1991). The murine promoter has also been utilized to express a number of heterologous proteins in the milk of transgenic mice. These include the oncogenes Ha-*ras* and c-*myc* (Andres *et al.*, 1987, 1988), human CD4 (Yu *et al.*, 1989), human growth hormone (Reddy *et al.*, 1991; Tojo *et al.*, 1993) and extracellular superoxide dismutase (SOD) (Hansson *et al.*, 1994b), while rabbit WAP has

been used to express a variant of human α_1AT (Bischoff *et al.*, 1992). The murine promoter has also been utilized to express human t-PA in both mice and goats (Gordon *et al.*, 1987; Pittius *et al.*, 1988; Ebert *et al.*, 1991), human protein C in pigs (Velander *et al.*, 1992) and human growth hormone in rats (Ninomiya *et al.*, 1994).

Studies of the gene elements responsible for the tissue-specific regulation of WAP expression have been done largely with transgenic mice. Intragenic sequences, as well as the 3' untranslated region of the WAP gene, appear to be crucial for mammary-specific and high-level expression of WAP in transgenic mice (Bayna and Rosen, 1990; Burdon *et al.*, 1991; Dale *et al.*, 1992). The murine WAP promoter has been utilized to express human t-PA in the milk of transgenic mice (Gordon *et al.*, 1987; Pittius *et al.*, 1988). This protein has clinical potential to dissolve fibrin clots in victims of myocardial infarction. The transgene consisted of 2.6 kb of the upstream WAP sequence, including the endogenous cap site, with t-PA cDNA cloned at nucleotide 24 so that this construct utilized the start point of translation and secretion signal sequence within the t-PA cDNA. The polyadenylation and termination signals downstream of the cDNA sequence were supplied from SV40. Expression ranged from <20 to 50 000 ng ml^{-1}, approximately 5% of the endogenous WAP level. The production of functional t-PA was confirmed by fibrin clot lysis and enzyme-linked immunosorbent assay (ELISA). The transgene was predominantly expressed in the mammary gland, but low levels of WAP–t-PA mRNA were detected in the tongue, kidney and sublingual gland (Pittius *et al.*, 1988). When this construct was introduced into goats, one transgenic line expressed enzymatically active t-PA at a concentration of 3–6 μg ml^{-1} (Ebert *et al.*, 1991).

A soluble derivative of human CD4 (hCD4), the human immunodeficiency virus receptor and a potential therapeutic agent for the treatment of AIDS, has also been expressed in the mammary gland of transgenic mice (Yu *et al.*, 1989). Expression levels ranged from 10 to 200 ng ml^{-1} as determined by immunoassay techniques. The WAP–hCD4 transgene contained the hCD4 cDNA plus 1850 bp of 5' upstream WAP sequence, and the bovine growth hormone polyadenylation signal and downstream region cloned 3' to the hCD4 cDNA.

Animals transgenic for human growth hormone are known to develop a number of abnormalities (Pursel *et al.*, 1989). However, human growth hormone has been expressed in the milk of transgenic mice with no adverse effects detected (Reddy *et al.*, 1991). The human growth hormone gene was expressed specifically in the mammary gland using the 2.6 kb murine WAP promoter. Expression levels varied greatly, from 65 ng ml^{-1} to 410 μg ml^{-1}. The recombinant protein was also detected in the serum during lactation; however, no adverse effects were detected. When homozygous mice were generated from the highest-producing line, levels of greater than 3.5 mg ml^{-1} were achieved. This compared favourably with the 1 mg ml^{-1} produced with mammalian tissue culture (Bebbington and Hentschel, 1985).

Generally, the expression levels of the WAP constructs have been very low. Except for the above case, the highest expression of a heterologous protein was only 50 μg ml^{-1} (Gordon *et al.*, 1987). These findings suggest that sequences within the WAP coding region or the 3' untranslated region (3'UTR) contribute to high-level expression. Comparisons between the mouse, rat and rabbit WAP genes have shown that the 3'UTR is more highly conserved than the protein-coding region (Campbell *et al.*, 1984; Thepot *et al.*, 1990). Dale *et al.* (1992) have determined by deletion studies that

the 3'UTR is indeed necessary for high-level expression of this transgene in mice. The site-independent, high-level expression of rat WAP has been achieved using only 949 bp of 5' and 70 bp of 3' flanking sequences. Expression levels were also found to be proportional to the transgene copy number. Replacement of the WAP 3'UTR with the corresponding SV40 downstream sequence resulted in reduced expression, while further deletion of 91 bp of the 3'UTR led to site-dependent expression. The importance of the 3'UTR for high-level expression may explain why the above WAP-based constructs (Gordon *et al.*, 1987; Pittius *et al.*, 1988; Yu *et al.*, 1989), lacking the WAP 3'UTR expressed so poorly.

Better WAP-driven expression of heterologous proteins has been obtained in pigs. The complete 7.2 kb murine WAP gene has been expressed in the milk of transgenic swine at a level of approximately 1–2 mg ml^{-1} in three different transgenic lines, 2 mg ml^{-1} being the endogenous expression level in mice (Wall *et al.*, 1991). This construct seemed to give consistent expression levels regardless of the site of integration, contrasting with the same transgene expressed in mice (Burdon *et al.*, 1991). These data indicate that the murine WAP promoter could be used to express valuable pharmaceutical proteins in the milk of transgenic pigs. To investigate this possibility, the cDNA sequence encoding human protein C, a potent anticoagulant, was cloned into the first exon of the above murine WAP gene. When introduced into swine, the recombinant product was produced at levels up to 1 mg ml^{-1} (Velander *et al.*, 1992). The recombinant human protein C demonstrated anticoagulant activity equivalent to that derived from human plasma, despite exhibiting variable levels of γ-carboxylation. The recombinant protein may be clinically useful in the treatment of victims of stroke, septic shock and those suffering from a congenital deficiency in protein C (Esmon, 1987). The projected US market value of protein C, according to the American Red Cross, is estimated at $US960 million (Pursel and Rexroad, 1993).

α-Lactalbumin

Guinea-pig and goat α-lactalbumin have been expressed in transgenic mice (Maschio *et al.*, 1991; Soulier *et al.*, 1992) while the bovine gene has been expressed in both mice and rats (Vilotte *et al.*, 1989; Hochi *et al.*, 1992). The bovine α-lactalbumin promoter has also been utilized to express ovine trophoblast interferon (oTP) in mice (Stinnakre *et al.*, 1991) as well as human growth hormone in rats (Ninomiya *et al.*, 1994).

The bovine transgene comprising the α-lactalbumin transcriptional unit (3.2 kb) with 750 bp upstream and 336 bp 3' flanking sequence was expressed in a tissue-specific manner in five out of six transgenic lines generated (Vilotte *et al.*, 1989). The best expressing line produced bovine α-lactalbumin at 0.45 mg ml^{-1}, which is equivalent to apprxoimately half the endogenous level expressed in the milk of both cows and mice. The same promoter has also been utilized to express oTP in the milk of transgenic mice (Stinnakre *et al.*, 1991). The transgene consisted of oTP cDNA cloned between the bovine α-lactalbumin 5' and 3' flanking regions. Out of the eight transgenic mice generated, only one transgenic line was found to express active oTP, and at a greatly reduced level of approximately 1 μg ml^{-1}.

Further transgenic expression studies have determined that as little as 477 bp of upstream sequence confers tissue-specific expression of the bovine α-lactalbumin

gene in transgenic mice, with important *cis*-acting elements located within the region –477/–220 (Soulier *et al.*, 1992).

Secretion of bovine α-lactalbumin into the milk of transgenic rats at levels higher than the endogenous bovine expression level has been obtained with a gene very similar to that expressed in mice (Hochi *et al.*, 1992). This construct contained approximately 800 bp of 5' sequence and 500 bp of downstream sequence, and was maximally expressed at 2.4 mg ml^{-1}, five times that reported in mice (Hochi *et al.*, 1992). Reasons for this dramatic increase in expression may be due to the different transcriptional machinery between the two host animals or to reported differences in the gene sequence (13 nucleotides different) or to a combination of these factors. The bovine α-lactalbumin promoter (738 bp) has also been utilized to drive the expression of human growth hormone at levels as high as 4.4 mg ml^{-1} in rats (Ninomiya *et al.*, 1994).

The goat α-lactalbumin gene (21 kb in length, 8.5 kb of which is 5' upstream sequence) has also been expressed in the milk of transgenic mice up to 3.7 mg ml^{-1}, nearly twice the endogenous level in goats and five times that in mice (Soulier *et al.*, 1992). However, the expression levels varied greatly between transgenic lines and were copy number-independent, indicating that some *cis*-acting regulatory elements were missing.

Mice deficient in α-lactalbumin (Stinnakre *et al.*, 1994), as well as mice that have had their endogenous gene replaced with that of human α-lactalbumin gene, have recently been generated (Stacy *et al.*, 1994). These experiments have special significance for the genetic engineering of milk composition, which will be discussed later.

β-*Casein*

Both rat and goat β-casein have been expressed in the milk of transgenic mice (Lee *et al.*, 1988; Persuy *et al.*, 1992; Roberts *et al.*, 1992). β-Casein promoters from various species have been utilized to drive the expression of a number of heterologous proteins, such as bovine follicle-stimulating hormone (bFSH) and hCFTR in mice (Greenberg *et al.*, 1991; DiTullio *et al.*, 1992), human growth hormone in rats (Ninomiya *et al.*, 1994), hIL-2 in rabbits (Bühler *et al.*, 1990) and ht-PA in goats (Ebert *et al.*, 1991).

The 14 kb genomic sequence containing the rat β-casein gene, as well as 3.5 kb of 5' and 3.0 kb of 3' flanking sequence, has been expressed in transgenic mice (Lee *et al.*, 1988). Eight transgenic lines were generated; however, mRNA expression studies determined that rat β-casein expression was extremely low, being only 0.01–1% of the endogenous mouse β-casein expression levels. These data indicate that important regulatory elements necessary for high-level expression of this transgene were missing from this construct. However, all the transgenic lines expressed in a tissue-specific manner except for one line, which also expressed β-casein mRNA in the brain, albeit at a much lower level.

Further studies with various rat β-casein–chloramphenicol acetyltransferase fusion genes have determined that a –524/+490 minimal promoter can direct heterologous expression to the mammary gland (Lee, Atiee and Rosen, 1989). This same promoter was utilized to produce two separate constructs containing cDNA sequences for the

bFSH subunits α and β respectively. Bigenic mice, generated by co-injection of both constructs, or by crossing individual lines expressing separate subunits, produced biologically active bFSH in their milk at levels up to 15 μg ml^{-1}. The presence of a biologically active product demonstrates that the mammary gland is able to synthesize and assemble bioactive heterodimeric proteins (Greenberg *et al.*, 1991).

The rabbit β-casein promoter has been used to express biologically active hIL-2 in rabbits at a maximal level of only 430 ng ml^{-1} (Bühler *et al.*, 1990). The transgene consisted of the 2 kb rabbit β-casein promoter cloned 5' to the genomic sequence of hIL-2. This construct contained vector sequences between the junction of these two genetic elements which may have contributed to its poor expression. Alternatively, it seems that β-casein transgenes require greater upstream sequence for high-level expression. For a review of the use of hIL-2 in immunotherapy, see Kaplan, Cohn and Smith (1992).

A dramatic improvement in expression levels, compared to the rat gene, was found with mice transgenic for the 18 kb caprine β-casein gene, containing 3 kb of 5' and 6 kb of 3' sequence (Persuy *et al.*, 1992). All of the nine transgenic lines expressed caprine β-casein in their milk, with two lines expressing as much as 21–24 mg ml^{-1}, twice the endogenous level in goats. A homozygous mouse expressed as much as 40 mg ml^{-1} (higher than the endogenous mouse β-casein expression level of 20–30 mg ml^{-1}). All the founder mice exhibited tissue-specific expression, while in two cases caprine β-casein mRNA was detected in the skin. This extremely high expression level indicates that the goat β-casein may be a useful promoter for the production of high-value bioactive proteins in the milk of transgenic animals. The goat β-casein promoter has been used to drive the expression of t-PA in the milk of transgenic goats at a level of 2–3 mg ml^{-1} (Ebert *et al.*, 1991, 1994).

The reason for the extremely low level of expression of the rat β-casein transgene with respect to the highly expressing goat equivalent is probably due to the divergence between the rat and goat β-casein genes (Persuy *et al.*, 1992). The rat and goat genes exhibit 75% homology over the first 200 bp of the 5' flanking region; however, beyond this point there is little homology between their upstream sequences (Roberts *et al.*, 1992).

The goat β-casein gene isolated by Roberts *et al.* (1992) has been used to drive the expression of heterologous proteins in the milk of both transgenic mice and goats (DiTullio *et al.*, 1992; Ebert *et al.*, 1991). A construct containing the cDNA sequence for hCFTR cloned between exons 2 and 7 of the goat β-casein gene (i.e. the cDNA sequence is flanked on either side by at least one intron) was microinjected into mice (DiTullio *et al.*, 1992). Transgenic expression was found to be specific to the mammary gland, recombinant hCFTR being associated with the membrane surrounding the milk fat globule. The authenticity of the protein product was confirmed by immunoprecipitation, but expression levels could not be accurately quantified. This construct has also been introduced into goats (H. Meade, personal communication). It is hoped that the production of recombinant hCFTR may help in the development of therapies for cystic fibrosis. The feasibility of utilizing hCFTR in protein replacement therapy has been demonstrated *in vitro* by Marshall *et al.* (1994).

α_{s1}-CASEIN

The bovine α_{s1}-casein promoter has been utilized to express a variety of recombinant human proteins in the milk of transgenic mice. These proteins include human urokinase (Meade *et al.*, 1990), lysozyme (Maga *et al.*, 1994) and lactoferrin (Platenburg *et al.*, 1994). Of the handful of transgenic cattle established, to date, two lines contain portions of the bovine α_{s1}-casein gene (Krimpenfort *et al.*, 1991; Hyttinen *et al.*, 1994).

Meade *et al.* (1990) constructed a mammary-specific expression vector consisting of 21 kb of 5' and 2 kb of 3' flanking sequence, including the native polyadenylation signal, of the bovine α_{s1}-casein gene joined by a synthetic linker containing sites for the rare cutting restriction enzymes, *Xho*I and *Not*I. The sequence encoding the genomic human urokinase gene was cloned into the *Xho*I site and the construct microinjected into the pronuclei of fertilized mice oocytes. Lactating transgenic mice secreted functional human urokinase in their milk at levels of 1–2 mg ml^{-1}. Analysis of RNA from various tissues demonstrated that human urokinase was expressed only in the mammary gland. The cysteine-rich human urokinase protein, which contains nine disulphide bonds, was specifically selected to investigate whether the mammary gland could synthesize proteins possessing a high proportion of sulphur-containing amino acids. The recombinant urokinase was demonstrated to be enzymatically active, as determined by casein/plasminogen gel assay (Nagamine, Sudol and Reich, 1983). It has been estimated that if the above construct functioned as well in transgenic cows, a single cow could produce approximately 20 g of this recombinant protein per day.

Maga *et al.* (1994) have used the same α_{s1}-casein promoter as above to express human lysozyme (cDNA sequence) in the milk of transgenic mice. Out of six transgenic lines established, two expressed human lysozyme RNA in the mammary gland, one line expressing more than the endogenous WAP RNA level. Protein expression studies have determined that as much as 0.78 mg ml^{-1} of active human lysozyme is expressed in the milk of these mice (J. Murray, personal communication).

Krimpenfort *et al.* (1991) have succeeded in producing a transgenic calf containing a transgene consisting of the human lactoferrin cDNA cloned between the second and second to last exons of the bovine α_{s1}-casein gene. Injection of over 1000 embryos resulted in the production of one female and one male transgenic calf. The female proved to be mosaic, and restriction enzyme analysis indicated that a portion of the transgene had been deleted. The male calf, 'Herman', contains 5–10 copies of the intact transgene in a head-to-tail arrangement. The production of lactating transgenic daughters from Herman will be necessary before expression studies can be performed. This may be as late as 1998. Expression studies in transgenic mice generated using a construct similar to the one described above have resulted in human lactoferrin expression levels of 0.1–36 μg ml^{-1} (Platenburg *et al.*, 1994). Higher expression levels were observed when the lactoferrin cDNA was replaced with the genomic sequence (De Wit and Pieper, unpublished).

Both lysozyme and lactoferrin have antimicrobial activity (Arnold, Cole and McGhee, 1977; Hughey and Johnson, 1987; Chang, 1990). Thus, the presence of these proteins within the milk of cattle should have the additional benefit of reducing intramammary infections, or mastitis, a major problem in the dairy industry which is

estimated to cost over $US100 million annually in lost production and treatment costs in New York State alone (Miles, Lesser and Sears, 1992).

A transgenic calf containing the human erythropoietin sequence under the control of the bovine α_{S1}-casein promoter has recently been generated (Hyttinen *et al.*, 1994). The transgene construct consists of bases –610 to +1483 of the bovine α_{S1}-casein gene with the genomic erythropoietin sequence cloned downstream. This construct also contains the 1.3 kb chicken lysozyme A element and the mouse mammary tumour virus hormone response element (bases 7112–7295) cloned upstream of the α_{S1}-casein promoter.

General comments on mammary-specific promoters

CROSSING SPECIES BOUNDARIES

The fact that many milk protein genes from one mammal can be expressed efficiently in the mammary gland of another indicates that the genetic elements that determine mammary-specific expression are often conserved between species (Palmiter and Brinster, 1986). In some cases transgene expression levels may even exceed those in the species of origin, oBLG expression in mice being an example (Simons, McClenaghan and Clark, 1987). Similar experiences have been reported for mouse WAP in swine (Wall *et al.*, 1991; Velander *et al.*, 1992), goat α-lactalbumin (Soulier *et al.*, 1992) and β-casein (Persuy *et al.*, 1992) in mice, as well as bovine α-lactalbumin in rats (Hochi *et al.*, 1992). Therefore, to drive the expression of foreign proteins in the milk of transgenic animals it may be advantageous to utilize promoters from a different mammalian species. This has been demonstrated with the high-level expression of human protein C in transgenic swine utilizing the mouse WAP promoter (Velander *et al.*, 1992), while the same construct was expressed at a reduced level in transgenic mice (Velander *et al.*, 1991).

WHEY VERSUS CASEIN PROMOTERS

As the caseins are expressed at higher levels than the whey proteins in the milk of ruminants, it might be expected that casein transgenes would express at higher levels than whey transgenes. This, however, is not generally the case. With the exception of goat β-casein (Persuy *et al.*, 1992), whey transgenes tend to express at a higher level than their casein counterparts in mice.

Transgenic experiments have demonstrated that whey protein genes possess more compact promoters and give better expression than the caseins. High-level expression has been reported in transgenic mice for bovine α-lactalbumin (Vilotte *et al.*, 1989), rat WAP (Dale *et al.*, 1992) and oBLG (Whitelaw *et al.*, 1992) with as little as 750, 949 and 406 bp of upstream sequence, respectively. In the case of the rat WAP and oBLG transgenes, expression is also copy number-dependent (Dale *et al.*, 1992; Whitelaw *et al.*, 1992). Thus, expression of these whey protein transgenes occurs in a copy number-dependent fashion and is impervious to positional effects, an effect normally only associated with the presence of DCR sequences. In contrast, position-independent, copy number-dependent expression for casein-based transgenic constructs has not so far been observed. These results indicate that the *cis*-acting elements necessary

to confer independent expression without interference from the transcriptional state of the surrounding chromatin are missing from these transgenes. An explanation for these findings may be that casein genes are naturally organized as a gene cluster and may be under the influence of a DCR similar to that discovered in the β-globin locus. In contrast, the whey protein genes are scattered throughout the genome and so their expression is independently regulated.

Ninomiya *et al.* (1994) have performed comparative studies of bovine casein transgenes in transgenic rats. This group has managed to achieve high-level expression of human growth hormone in transgenic rats using as litle as 671 bp and 1.7 kb of bovine α_{S1}-and β-casein upstream sequence, respectively. Although high-level expression was achieved, expression levels were still highly variable and subject to positional effects (87 μg ml^{-1} to 6.5 mg ml^{-1} and 1 μg ml^{-1} to 10.9 mg ml^{-1}, respectively). The presence of a DCR in the casein locus has not been identified.

THE EFFECT OF INTRONS

When designing constructs for the production of foreign proteins in the milk of transgenic animals, the protein coding region may be present as either the cDNA or genomic sequence. Generally higher expression levels, of the order of mg ml^{-1}, have been achieved with genomic sequences (Archibald *et al.*, 1990; Meade *et al.*, 1990; Wright *et al.*, 1991), than constructs utilizing cDNA coding sequences (Pittius *et al.*, 1988; Simons *et al.*, 1988; Clark *et al.*, 1989b; Yu *et al.*, 1989; Ebert *et al.*, 1991; Greenberg *et al.*, 1991; Stinnakre *et al.*, 1991). A study by Brinster *et al.* (1988) using four different pairs of constructs, each pair differing solely in the presence or absence of introns in their protein coding region, determined that transgenes containing introns generally produced 10- to 100-fold more mRNA transcripts than those that lacked introns. These findings may be due to the presence of enhancers or other *cis*-acting sequences necessary for high-level expression being located within introns (Brinster *et al.*, 1988). Indeed, regulatory elements have been identified within the intronic sequences of the human adenosine deaminase gene and the human hypoxanthine phosphoribosyltransferase gene (Aronow *et al.*, 1989; Reid *et al.*, 1990). This may also be the case with milk protein genes (Archibald *et al.*, 1990; Whitelaw *et al.*, 1991). Findings similar to those of Brinster *et al.* (1988) have been reported using the oBLG transgene in mice (Whitelaw *et al.*, 1991). The question arises as to whether this augmentation effect is due to intron sequences required for efficient splicing of the mRNA transcript or whether the absence of introns removes important genetic regulatory elements. Experiments reported by Whitelaw *et al.* (1991) suggest that it is, in fact, sequences within the introns themselves that regulate gene expression in concert with the 5' oBLG flanking sequence.

Complementary DNA sequences have frequently been used in transgenic constructs as they are usually the first form of the gene available and are easier to manipulate and clone compared to genomic sequences, which may be many kilobases long. In cases where only the cDNA of the gene of interest is available, or the genomic sequence is difficult to manipulate, studies have demonstrated that the addition of heterologous introns may improve their expression (Palmiter *et al.*, 1991; Whitelaw *et al.*, 1991; Shani *et al.*, 1992). Shani *et al.* (in preparation) have demonstrated that this augmentation effect of introns on gene expression can function synergistically

with constructs containing different combinations of introns expressing at different levels. A variety of oBLG–hSA minigenes containing combinations of hSA introns 1–14 have been constructed and expression studies performed in tissue culture using COS cells. Various combinations of introns were found to give efficient expression. Similar constructs used to generate transgenic mice have also been observed to give good expression, up to 10 mg ml^{-1} (M. Shani, personal communication).

IMPROVING TRANSGENIC EXPRESSION

Two methods have been demonstrated to improve transgene expression. Both methods rely on creating a permissive region of transcription around the site of integration. This permissive region shields the transgene from the effects of the transcriptional state of the surrounding host chromatin which may repress transgene expression.

'Rescuing' transgene expression

The expression of poorly expressed transgenes can be elevated via co-integration with a highly expressing construct. This effect has been demonstrated by co-injecting the low expressing cDNA-based constructs, oBLG–FIX and oBLG–α_1AT (described above), with the high expressing oBLG transgene in mice (Clark *et al.*, 1992). The oBLG–FIX construct did not express at all in 10 different transgenic mouse lines established, but when co-integrated with the oBLG transgene, FIX expression levels were elevated to a maximum of 1 μg ml^{-1}. Similar results were found with the oBLG–α_1AT construct (up to 600 μg ml^{-1}). Clark *et al.* (1992) proposed that the highly expressing oBLG gene, which has co-integrated with the cDNA-based construct, is able to establish an active transcriptional locus, thereby overcoming the position effects responsible for the low expression of the cDNA-based constructs. This 'rescue' technique may be applicable to other low-expressing cDNA-based constructs.

Utilizing MAR sequences

The co-integration of matrix attachment regions (MAR) has been demonstrated to confer position-independent expression of murine WAP in transgenic mice (McKnight *et al.*, 1992). These MAR sequences from the chicken lysozyme locus restored normal hormonal and lactational regulation and are thought to buffer the transgene from positional effects which tend to interfere with transgene expression. In this study all WAP transgenes which had integrated next to a MAR sequence were actively transcribed. However, only 50% of the transgenes that integrated without MAR sequences were actively transcribed.

In studies performed with pigs and sheep, generally about 60% of the transgenic animals generated actively express foreign transgenes (Pursel *et al.*, 1990; Rexroad *et al.*, 1990; McKnight *et al.*, 1992). It may be possible to use MAR sequences to increase this proportion of transgenic expressors, and therefore reduce production costs. Such a sequence has been utilized in the α_{S1}-casein–human erythropoietin construct introduced into cattle by Hyttinen *et al.* (1994).

Genetic engineering of bovine milk composition

Another application of transgenic technology of interest to the dairy and related industries is the alteration of milk composition in dairy animals. Using a combination of recombinant DNA techniques and transgenics it should be possible to modify milk composition, thereby allowing the production of novel milk products.

Possible ways in which the composition of bovine milk might be altered include:

1. '*humanizing*' the milk of cows to make it more suitable to the nutritional needs of human infants;
2. the production of '*designer*' milks for those individuals unable to tolerate certain milk components;
3. the alteration of the casein content of cow's milk for improved nutrition and greater cheese yields; and, finally,
4. engineering milk proteins with altered physical and chemical properties for specific manufacturing processes.

The above aims could be realized in some cases by adding extra milk protein genes, via micro-injection, to the germline of dairy animals. For example, introducing a transgene encoding a human milk protein to the germline in order to make cow's milk closer in composition to human milk. In other cases it would be desirable to down-regulate the expression of certain endogenous milk protein genes which have been implicated in allergic reactions in some individuals. It has been proposed that milk with little or no lactose could be produced by down-regulating the α-lactalbumin gene or by the introduction into dairy cattle of the β-galactosidase transgene (Bremel, Heng-Cherl and Bleck, 1989). A study conducted by the US National Dairy Council has shown that approximately 70% of the world's population is deficient in the intestinal lactase necessary to digest the lactose present in milk (NDC, 1985). Adults exhibiting severe lactose intolerance may suffer from cramps, flatulence and diarrhoea, while children may suffer malnutrition or even death in extreme cases (Kretchmer, 1972; Saavedra and Perman, 1989). It is possible to selectively disrupt or entirely remove specific genes by utilizing 'knock out' technology via homologous recombination in ES cells (Cappecchi, 1989; Hasty *et al.*, 1991; Valancius and Smithies, 1991). Stinnakre *et al.* (1994) have succeeded in producing α-lactalbumin-deficient mice via gene disruption using homologous recombination in ES cells. Lactating transgenic females homozygous for this null mutation synthesize a milk deficient in α-lactalbumin and lactose. This milk is higher in fat and protein; however, the milk is so viscous that it cannot be secreted. This is probably due to the fact that lactose is the major osmole of bovine milk, and altering its levels in the mammary gland may have interfered with osmoregulation (Kuhn, 1983). Hemizygous females exhibited a 40% decrease in α-lactalbumin levels and only a 10–20% reduction in lactose levels compared to wild type.

An alternative approach to the specific down-regulation of milk protein genes may be the use of antisense and ribozyme sequences which are able to suppress native gene expression by blocking the flow of information from DNA to RNA to protein (reviewed by Inouye, 1988; Cazenave and Hélène, 1991; Symons, 1992; Bratty *et al.*, 1993).

Antisense technology offers the ability to down-regulate selectively a specific gene

by utilizing RNA molecules complementary to the protein-coding, i.e. sense, mRNA sequence. Antisense molecules base-pair with the target RNA forming a double-stranded structure which hinders the binding of intracellular factors essential for translation (Cazenave and Hélène, 1991).

Antisense transgenes can be constructed by cloning the target sequence in reverse orietnation, under the regulation of an appropriate promoter. The successful application of antisense technology to down-regulate gene expression has been demonstrated dramatically by the production of transgenic dwarf mice by introducing a transgene containing the antisense cDNA sequence of rat growth hormone (Matsumoto *et al.*, 1993). A model system involving chloramphenicol acetyltransferase (CAT) expression driven by bovine α_{s1}-casein promoter has also given encouraging results in that the expression of the CAT gene in the mammary gland was reduced by 92% in bigenic mice containing the antisense CAT sequence under the same α_{s1}-casein promoter (Sokol *et al.*, submitted).

Ribozymes are RNA species that are able to cleave specific RNA sequences catalytically by virtue of their endoribonuclease activity. Target specificity is dictated by the antisense RNA sequence flanking the catalytic hammerhead domain via Watson–Crick base-pairing with the complementary substrate sequence (Haseloff and Gerlach, 1988). Since ribozymes should be able to act on many target RNAs, they should, theoretically, achieve greater down-regulation of gene expression than antisense RNA, although it is not clear that this occurs *in vivo*. Synthetic ribozymes have been used to down-regulate gene expression *in vivo* in bacteria (Sioud and Drlica, 1991), mammalian cells (Cameron and Jenings, 1989; Sarver *et al.*, 1990; Scanlon *et al.*, 1991), *Xenopus* oocytes (Cotten and Birnsteil, 1989; Saxena and Ackerman, 1990), plant cell protoplasts (Steinecke, Hergert and Schreier, 1992), *Drosophila* (Zhao and Pick, 1993) and the murine mammary gland (Sokol *et al.*, submitted).

In some cases, it may be advantageous to modify the amino acid sequence of the milk protein genes to engineer altered properties, such as proteins with increased nutritional value (Oh and Richardson, 1991a) or enhanced gelation characteristics (Lee, Cho and Batt, 1993). The technique of *in vitro* site-directed mutagenesis (Zoller and Smith, 1983; Botstein and Shortle, 1985; Jimenez-Flores and Richardson, 1988) allows the systematic modification of the amino acid content of any cloned protein-encoding sequence, thus permitting the rational design of food proteins with more desirable physical and chemical properties.

'HUMANIZING' COW'S MILK

Despite the superiority of the breast milk of healthy mothers over that of cow's milk-based substitutes, many mothers are either unable or choose not to breast feed their children. As a result, many infants throughout the world are raised on supplemented formulae based on cow's milk (Packard, 1982).

There are some important compositional differences between bovine and human milk. Most notable is the greater whey to casein ratio, a higher concentration of both lactoferrin and lysozyme, and the absence of BLG in human milk (Blanc, 1981).

Bovine milk alone is insufficient for the nutritional needs of growing infants, thus requiring milk formula manufacturers to develop fortified 'humanized' cow's milk-based formulae to simulate human milk (Packard, 1982). Transgenic technology may be able to achieve this via a combination of down-regulating certain bovine milk

genes and/or by directing the expression of human milk protein transgenes to the bovine mammary gland (Mercier, 1986). An example is the attempt to introduce human lactoferrin into the milk of transgenic cattle (Krimpenfort *et al.*, 1991). Ward *et al.* (1992) and Liang and Richardson (1993) have succeeded in producing recombinant human lactoferrin in *Aspergillus oryzae* and *S. cerevisiae* at a level of 25 $\mu g\ ml^{-1}$ and 2 $\mu g\ ml^{-1}$ of culture media, respectively. Lactoferrin from these sources could be used in the production of supplemented infant formula. The infant formula market is valued at over $US4 billion per year (Spalding, 1992).

As mentioned above, BLG is the major whey protein of cow's milk, yet it is absent from that of humans. Since bovine milk is a major component of the Western diet, the proteins of cow's milk are usually the first foreign proteins to be encountered by human infants (Swaisgood, 1973). Therefore cow's milk can elicit an allergic response, resulting in gastrointestinal, cutaneous and respiratory hypersensitivity conditions in as many as 7.5% of newborns (Packard, 1982). BLG in particular has been reported to be one of the main agonists in cow's milk allergy in infants, and increased levels of BLG antibodies have been reported in sufferers of cow's milk protein intolerance (James and Sampson, 1992). Recently, BLG has also been implicated in interfering with the development of the newborn's gut mucosal barrier (Arvola *et al.*, 1993). For these reasons methods have been developed to remove BLG from bovine milk destined for consumption by infants (Kuwata *et al.*, 1985). Transgenic cattle deficient in BLG expression are another alternative. This end could be achieved by introducing transgenic constructs containing antisense and/or ribozyme sequences directed against BLG to down-regulate its native expression levels.

Human milk has a lower casein:whey ratio than cow's milk. Its protein fraction contains only 30% casein, mostly β-casein, compared to 80% casein in the cow, where α_{S1}-casein is the predominant type (Jenness and Sloan, 1970). Down-regulation of α_{S1}-casein expression in cows, using methods described above, would lower the casein:whey ratio in cow's milk. β-Casein would become the dominant casein species in cow's milk, resulting in a milk that more closely resembles that of humans.

ENGINEERING OF MILK PROTEINS

Altering casein levels for improved milk properties

We will now consider aspects of modifying the casein content of cow's milk for improved functionality with respect to the dairy processing industry. This may be achieved by the addition of extra copies of either native or modified bovine casein genes to the genome of dairy cattle. Increasing the level of a single native casein species can alter milk functionality and thus enhance its utility in various dairy processes (Yun, Ohmiya and Shimizu, 1982; Pearse *et al.*, 1986; Jimenez-Flores and Richardson, 1988; Hennighausen, Ruiz and Wall, 1990).

Adding extra casein genes to the bovine genome may increase the protein content of milk, thereby increasing its nutritional value. There could also be considerable advantages for the cheese industry. Cheese yield, which is dependent on casein content, would also increase (Richardson, 1985; Kang, Jimenez-Flores and Richardson, 1986). It has been estimated that an increase of just 20% in the α_{S1}-casein content of milk would result in an extra $US190 million per year for the US dairy industry (Hennighausen, Ruiz and Wall, 1990).

The casein proteins in milk are predominantly present as a colloidal aggregate complexed with calcium phosphate to form spherical structures that are referred to as casein micelles (McMahon and Brown, 1984). On the removal of calcium, the casein micelles dissociate to form smaller non-colloidal protein complexes termed submicelles. The casein micellar structure is stabilized by an outer layer of κ-casein (Holt and Dalgleish, 1986). Waugh and Talbot (1971) have demonstrated that there is an approximately linear relationship between the surface area of artificial casein micelles and their κ-casein content. It has been proposed that cattle expressing higher concentrations of κ-casein in their milk would therefore contain smaller micelles. Indirect evidence to this effect has been obtained in our laboratory (Pearse *et al.*, 1986). Such milk should possess altered physical properties, such as enhanced thermal stability, which would be advantageous in reducing the gelation and coagulation properties of milk during heat-mediated sterilization (Jimenez-Flores and Richardson, 1988).

Cleavage of κ-casein by chymosin leads to the destabilization of the micelle and the precipitation of the caseins, the initial step in cheese-making (Delfour *et al.*, 1965). Milk with reduced levels of κ-casein would be expected to contain a greater proportion of larger micelles with reduced stability, and therefore would be more amenable to cheese production (Jimenez-Flores and Richardson, 1988).

Experiments to determine the effects of varying the concentration of various casein species, within artificial micelle milk, on rennet coagulation time (rate of curd formation) and syneresis (expulsion of whey from the curd) have been performed (Pearse *et al.*, 1986). It was found that increasing the concentration of both κ-casein and β-casein individually had the effect of reducing rennet clotting time. Higher levels of β-casein had the additional effect of an increased rate and extent of syneresis. The qualification here is the extent to which artificial micelles accurately mirror the *in vivo* situation. Yun, Ohmiya and Shimizu (1982) have also demonstrated that the addition of β-casein to milk can increase the rigidity of the curd, which increases the yield of milk solid that can be used in the cheese manufacturing process.

Engineering the caseins for improved cheese production

In recent years *in vitro* mutagenesis has been applied to the bovine casein genes to engineer proteins with altered amino acid sequences which may possess more properties beneficial for the cheese manufacturing industry. This research has centred chiefly on modifying the various peptide bonds cleaved by chymosin, either to increase the rate of hydrolysis for more economical cheese production (Oh and Richardson, 1991b), or to eliminate the production of undesirable peptides that affect the quality of the final product (Simons *et al.*, 1993).

Chymosin-mediated cleavage of bovine κ-casein occurs at the Phe–Met peptide bond (Delfour *et al.*, 1965). Site-directed mutagenesis of this Phe–Met bond to Phe–Phe has resulted in a κ-casein species which is cleaved at a higher rate by chymosin (Oh and Richardson, 1991b). Substantial economic benefits could be obtained if this modified casein was introduced into milk destined for cheese production.

Storage accounts for approximately 6–8% of the cost of cheese production in the UK. If methods could be developed to shorten the ripening time of cheese, without

adversely affecting flavour and texture, this would constitute quite a saving for the cheese industry (Law, 1987). The process of cheese maturation depends on the degradation of α_{s1}-casein via residual chymosin in the curd, which hydrolyses different peptide linkages at different rate (Creamer and Olson, 1982). The Phe23–Phe24 or Phe24–Val25 bonds in α_{s1}-casein are hydrolysed relatively quickly, while others, such as Leu149–Phe150, are hydrolysed more slowly, thus prolonging the cheese maturation process. It may be possible to engineer caseins with properties more amenable to cheese production using *in vitro* site-directed mutagenesis.

The proteolytic cleavage of β-casein by chymosin is usually slower than that of α_{s1}-casein (Grappin, Rank and Olson, 1985). The cleavage of the Leu192–Tyr193 peptide bond in β-casein results in the production of the C-terminal peptide (amino acids 193–209) responsible for the bitter taste defect in Gouda and other cheese varieties (Creamer, Mills and Richards, 1971; Visser and Slangen, 1977; Carles and Ribadeau-Dumas, 1984). Simons *et al.* (1993) have used site-directed mutagenesis to alter this Leu192–Tyr193 peptide bond to Pro192–Pro193 and Leu192–stop, resulting in an altered β-casein lacking this chymosin cleavage site.

Engineering the caseins for greater nutritional value

Although milk is almost a complete food in itself, the bovine caseins are relatively low in the sulphur-containing amino acids methionine and cysteine. The percentage of sulphur-containing amino acids present in the caseins could be increased via site-directed mutagenesis and then introduced into the bovine genome. A sulphur-enriched κ-casein has already been engineered to contain three extra methionine residues between Ala167 and Thr168 of the bovine κ-casein gene and has been expressed in *E. coli* at a level of 2 mg ml^{-1} (Oh and Richardson, 1991a).

As mentioned above, the down-regulation of BLG gene expression would aid in making the milk of cows more like that of humans. It has been postulated that down-regulation of the whey proteins would also result in increased expression of the casein genes. This rationale is based on the observation that cows homozygous for the B allele of BLG produce less BLG than the AA genotype and also produce 7% more casein (McLean, Graham and Ponzoni, 1984; Hill, 1993). This implies that the higher level of BLG production in the AA genotype is at the expense of casein production. It has been proposed that by eliminating the competition from BLG, via its genetic down-regulation, casein production may increase, making cow's milk more nutritious and of greater use to the cheese industry (Hill, 1993).

Protein engineering of the whey proteins

Recombinant bovine BLG has been expressed in *E. coli* as a preliminary to performing site-directed mutagenesis to design and produce modified BLG molecules for specific applications within the food industry (Batt *et al.*, 1990).

BLG possesses a free thiol group buried within the interior of its globular structure (Cys121). Upon heating, BLG unfolds exposing this cysteine residue, and so is then able to form aggregates with other BLG molecules and form gels (Sawyer, 1968). Upon heat treatment of milk, this free thiol group is also able to interact with the single disulphide group of κ-casein. This may interfere with the chymosin-mediated

hydrolysis of this protein critical for curd formation and cheese production (Morr, 1985). The heat-induced denaturation of BLG may also lead to the fouling of dairy processing equipment (Lalande, Tissier and Corrieu, 1985). The production of milk deficient in BLG may overcome this problem (Jimenez-Flores and Richardson, 1988). Alternatively, the substitution of extra cysteine residues proximal to the native free thiol at Cys121 (Leu104 to Cys104 or Ala132 to Cys132) has resulted in recombinant BLGs that are more resistant to thermal aggregation, presumably due to the formation of an additional disulphide linkage with Cys121 (Cho *et al.*, 1994).

Recombinant BLGs engineered to contain an extra free thiol group within the hydrophobic region of the molecule (Arg40 to Cys40, or Phe82 to Cys82) have demonstrated enhanced gelation characteristics (Lee, Cho and Batt, 1993). Additionally, a thermolabile recombinant BLG variant R40C/F82C (Arg40 to Cys40 and Phe82 to Cys82) has been demonstrated to reduce whey syneresis and time for curd formation when utilized in yoghurt production (Lee *et al.*, 1993).

The process of pasteurization destroys much of the lactoferrin present in milk. Recombinant human lactoferrin has been expressed in yeast with a view to increasing its heat stability via *in vitro* site-directed mutagenesis similar to that described above for BLG (Liang and Richardson, 1993).

Outlook/future prospects

TOWARDS THE MARKETPLACE

At present, a number of pharmaceutically active human proteins have been successfully produced in the milk of transgenic animals at commercially viable levels (*Table 6*). In five cases (human α_1AT, t-PA, growth hormone, serum albumin and protein C), recombinant protein production exceeds those reported for rival systems. In less than a decade, gene 'pharming' has established itself as a competititve alternative for the production of recombinant proteins. In the case of α_1AT, t-PA and protein C, transgenic expression has been established within the mammary gland of large farm animals (sheep, goats and pigs, respectively) allowing the harvesting of large quantities of recombinant protein from each animal. Once recombinant proteins from transgenic animals are approved for clinical use, it is expected that these products will become serious competitors for the world pharmaceutical market which is valued at many hundreds of millions of dollars (see *Table 6*).

The production of α_1AT in transgenic sheep provides a good case study. α_1AT deficiency affects approximately 100 000 people world-wide and leads to the onset of the degenerative lung disease, pulmonary emphysema (Crystal, 1989). Currently, the α_1AT needed to treat deficiency disorders is derived from human plasma; however, this source cannot provide the large quantities needed to treat these patients who require approximately 200 g α_1AT/patient/year (Casolaro *et al.*, 1987). A similar situation exists for protein C which, due to its very low serum concentration, is extremely difficult to isolate from human plasma (Velander *et al.*, 1991). Hence an alternate source of these clinically important proteins is needed. Recombinant α_1AT has been expressed in yeast (Cabezón *et al.*, 1984), though this non-glycosylated product has only 2% of the *in vivo* half-life of the plasma-derived product (Casolaro *et al.*, 1987). As described above, Wright *et al.* (1991) have succeeded in producing

Table 6. A comparison of pharmaceutically important human proteins expressed in the mammary gland of transgenic animals compared with expression levels in alternate systems

Protein	Biological function/ clinical utility	Expression levels[§]	World-wide sales ($US)	Reference
FIX	Blood clotting factor	25 ng ml^{-1}	$25 000/g*	Clark *et al.* (1989b)
	haemophilia B treatment	(100 μg ml^{-1}, only 2% active)		Brownlee (1987)
α_1-antitrypsin	Neutrophil elastase	7 mg ml^{-1}	$100 m[‡]	Archibald *et al.* (1990)
	inhibitor/emphysema	(60 ng ml^{-6} cells day^{-1})		Pavirani *et al.* (1989)
Interleukin-2	Cancer, AIDS and	430 ng ml^{-1}	$20 m[†]	Bühler *et al.* (1990)
	leprosy therapy	(10 μg ml^{-1} day^{-1})		Ryll *et al.* (1990)
t-PA	Thrombolytic agent	3 mg ml^{-1}	$230 m[†]	Ebert *et al.* (1991)
	myocardial infarction	(460 μg ml^{-1}.)		Datar, Cartwright and Rosen (1993)
Growth hormone	Hypopituitary dwarfism	11 mg ml^{-1}	$575 m[†]	Ninomiya *et al.* (1994)
	chronic renal insufficiency	(200 μg ml^{-1}.)		Friedman *et al.* (1989)
Protein C	Haemostasis regulator	1.0 mg ml-1	$960 m[ƒ]	Velander *et al.* (1992)
	stroke, septic shock	(<0.4 μg ml^{-1} h^{-1})		Velander *et al.* (1991)

[§] Expression levels obtained in the mammary gland of transgenic animals. Values in brackets are production levels achieved by microbial fermentation or mammalian cell culture.
[*] Clark *et al.* (1987).
[‡] Spalding (1992).
[†] World-wide sales values stated in $US millions for 1992 as given in Klauser (1993).
[ƒ] American Red Cross projection of US sales as quoted in Pursel and Rexroad (1993).

a transgenic sheep that secretes fully glycosylated recombinant α_1AT in its milk at a level of approximately 35 g l^{-1}, making α_1AT the most abundant milk protein. It has been estimated that such sheep producing α_1AT could produce more than 10 kg of recombinant protein per lactation (Wright *et al.*, 1991). The world-wide market for α_1AT has been estimated to be approximately $US100 million (Spalding, 1992).

THE COST EFFECTIVENESS OF GENE PHARMING

Not only do some transgenic animals express pharmaceutically active proteins at a higher level than competing systems, but gene pharming techniques are also more cost effective when compared to conventional fermentation technology. As an example of the expense involved in producing these products using established technology, Datar, Cartwright and Rosen (1993) have critically evaluated the process economics involved in the animal cell (CHO) and bacterial fermentation processes required for the production of recombinant t-PA. Their study estimates that the fermentation tanks alone needed to culture the CHO cells cost over $US6 million as an initial investment, with an annual expenditure of an additional $US23 million simply for fermentation materials. Recombinant t-PA produced by transformed *E. coli* is synthesized in a denatured form, necessitating an additional capital investment of over $US53 million to purchase the refolding tanks required to renature the recombinant protein and restore it to a functional state (Datar, Cartwright and Rosen, 1993).

In the past a major criticism of gene pharming has been the expense of producing transgenic animals. However, the cost of producing a founder transgenic pig, goat or cow has been estimated to be as low as $US25 000, $US60 000 and $US500 000, respectively (Hennighausen, 1990). Once the capital investment necessary to build and maintain a commercial fermentation plant is compared to the costs involved in transgenic animal production, gene pharming starts to look very competitive. The higher expression levels and lower production costs of transgenic bioreactors should translate into greater product availability and lower prices for the consumer, that is the patient.

A major factor contributing to the cost of transgenic animal production is the low efficiency of transgenesis, necessitating a large number of foster mothers to bear offspring, only a small percentage of which will be transgenic. For example fewer than 1% of micro-injected sheep, pig or cow embryos develop into transgenic offspring (Murray *et al.*, 1989). The number of animals and the effort required to produce transgenic animals could be reduced by screening for integration of the transgene early in embryo development via PCR, before implantation into a foster mother (Ninomiya *et al.*, 1989; Horvat *et al.*, 1993; Bowen *et al.*, 1994). This approach would decrease the number of non-transgenic pregnancies and therefore production costs. It has already been utilized by Hyttinen *et al.* (1994) in the generation of transgenic cattle. An additional practical advantage of utilizing animals as bioreactors is that their maintenance costs are much lower than those for a fermentation plant (Datar, Cartwright and Rosen, 1993).

A major disadvantage with fermentation processes are 'scale-up' problems, which may be experienced when progressing from pilot research reactors to large-scale commercial plants (Rhodes and Birch, 1988; Reisman, 1993). Scale-up often results in unpredictable alterations in productivity, usually with very uneconomical consequences (Reisman, 1993). However, scale-up problems can be avoided in gene

pharming as transgenic bioreactors are able to reproduce themselves while still retaining similar production characteristics from generation to generation (Carver *et al.*, 1993; Ebert *et al.*, 1994).

REGULATORY ISSUES AND MARKET ACCEPTANCE

As we have seen in this review, the cloning and characterization of the milk protein encoding genes has led to an avalanche of research within the past 10 years in utilizing these sequences to direct the expression of high-value pharmaceutical proteins to the mammary gland of various mammals. At present none of these recombinant proteins has appeared on the market, although α_1AT is expected to enter clinical trials in mid-1995 (Gordon Wright, personal communication).

One of the largest regulatory issues facing the use of recombinant proteins for human therapeutic use is that of product purity. Pharmaceutical proteins produced by recombinant DNA technology must be purified to remove all non-human proteins and other contaminants that may cause adverse reactions in patients (Anicetti, Keyt and Hancock, 1989). In cases where the recombinant protein constitutes a high proportion of the milk protein content, such as α_1AT expression in sheep, problems with product purification should be minimized. In addition, high expression levels in transgenic animals should allow more cost-effective protein purification than isolation from lower expressing fermentation systems, as the cost of purification is generally related to the product concentration (Wilkins and Velander, 1992). Indeed, recombinant t-PA has been obtained at greater than 98% purity from the milk of transgenic goats using a combination of acid fractionation, hydrophobic interaction and immunoaffinity chromatography (Denman *et al.*, 1991). Recombinant α_1AT derived from transgenic sheep has now been purified to greater than 99%, and will be the first protein derived from transgenic technology to be scrutinized by regulatory authorities (Gordon Wright, personal communication).

Altered milk products may also be just around the corner. For example, milk enriched with human lactoferrin destined for the production of infant formula (Krimpenfort *et al.*, 1991). As recombinant milk proteins produced in cattle are classified as food ingredients, they do not have to be exhaustively purified as do pharmaceutical proteins. Once the modified product is demonstrated to be safe for human consumption (through dietary-toxicity trials), approval by the relevant regulatory authorities is expected to be less costly and lengthy than that for recombinant proteins destined for pharmaceutical use (Spalding, 1992).

However, demonstrated product efficacy, safety and approval by the relevant regulatory authorities does not guarantee total market acceptance. Such is the case with recombinant bovine somatotropin (BST), a hormone used to increase milk production in dairy cattle, which has recently been approved for use in the USA. Although milk produced from cows treated with BST has been demonstrated to be completely safe and indistinguishable from that derived from other cows, the use of this recombinant product is facing opposition by various lobby groups in the USA (Hoyle, 1994). A study performed by Fox *et al.* (1994) indicates that the presentation of product information can greatly affect the way in which a recombinant product is perceived. For example, they found that students in urban California, who had not heard of BST before the study, initially showed a markedly negative bias against the

product. However, after being presented with a balanced appraisal of the product, approximately 70% demonstrated acceptance. This bias, however, was difficult to overcome in areas that had been subjected to campaigns against the use of recombinant BST. Therefore, education may be the key to successful marketing of these genetically altered food products. It may reasonably be expected that any public opposition to the use of therapeutic proteins derived from transgenic technology will be neutralized by the benefits that these drugs will provide for patients.

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