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14.1 Introduction

During lactation, mammary epithelial cells secrete large quantities of milk proteins. More than 90 % of these proteins are derived from the transcription of a few tissue-specific genes, expression of which is under a complex multi-hormonal regulation that involves both transcriptional and post-transcriptional mechanisms. Furthermore, to fulfil its bioreactor activity, the mammary gland needs an optimal supply of amino acids as well as efficient translation and transport machineries during lactation.

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The previous edition of this chapter (Vilotte *et al.*, 2002) described in detail the hormonal regulation of milk protein gene expression, their mRNA and gene structures, their co- and post-translational modifications and the transport and the secretion of milk proteins. The aim of this revision is to summarize briefly our knowledge on the structure of the milk protein genes and to put into context the rapid growth of information on the regulatory elements involved in controlling the expression of these genes. We will also focus on the amino acid supply to the mammary gland and on the intracellular routing and sorting of milk proteins in mammary cells. However, other important topics will not be discussed. The widespread presence of caseins variants will be covered in Chap. 15, while the practical applications of these studies for the dairy field will be described in Chap. 16. Similarly, global analysis of genome evolution with regard to the mammary gland, as described in Lemay *et al.* (2009), will be discussed in other chapters of this book.

14.2 Structure of Milk Protein Genes

The major milk protein genes have been sequenced in several species. Overall, the mosaic structure of these genes has been well conserved during evolution, and observed species differences in the length of their transcription unit can often be attributed to the occurrence of repetitive DNA within some introns, mainly artiodactyl

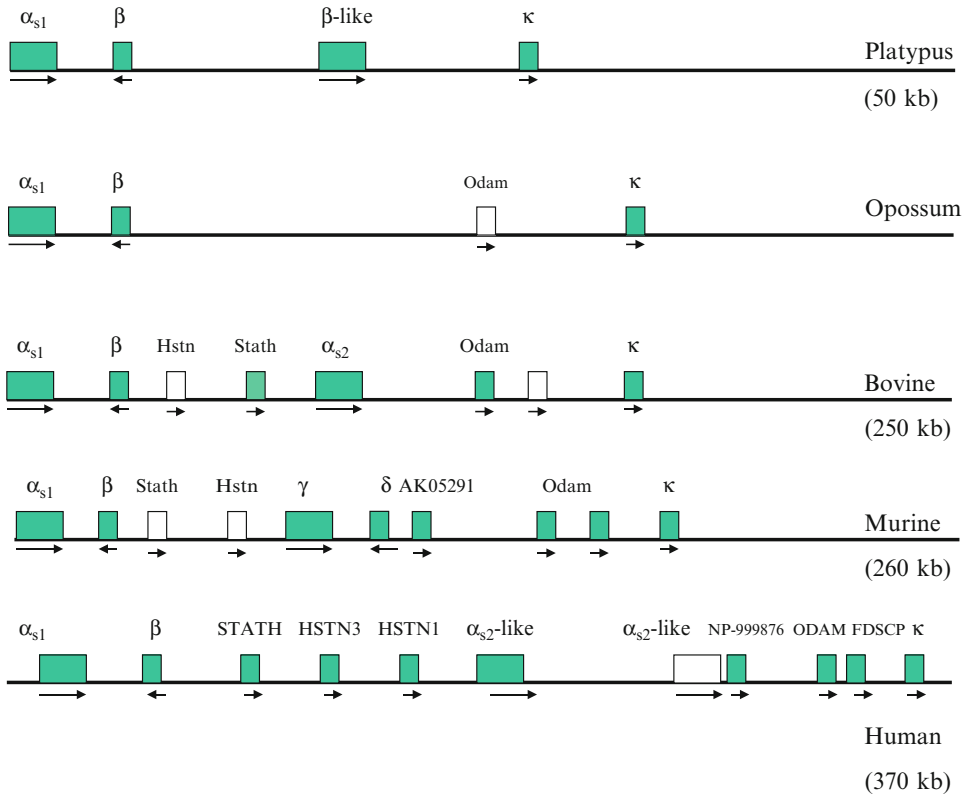


Fig. 14.1 Overall organization of the casein locus in various species. Green boxes represent the transcription unit of the genes. White boxes: putative genes. When

available, orientation of the gene transcription unit is indicated by an arrow. Origins of the data are mentioned in the text

retrotransposons. Similarly, deletions or insertions of amino acids between caseins from different species or variants appear to occur mostly by exon skipping. These genes share the canonical structure of tissue-specific eukaryotic genes. Beyond these basic similarities, these genes differ substantially in their genomic organization.

14.2.1 The Casein-Encoding Genes

14.2.1.1 General Organization of the Gene Cluster

Classical genetic studies have demonstrated that the four casein genes are closely linked, and a general organization of the gene cluster was deduced (reviewed by Grosclaude, 1979). Since then, structural analysis of the gene cluster at the DNA level in various species has confirmed and refined the protein data (Fig. 14.1; Tomlinson

et al., 1996; George *et al.*, 1997; Fujiwara *et al.*, 1997; Rijnkels *et al.*, 1997a,b,c; 2002; Lefèvre *et al.*, 2009). The overall size of the casein locus varies from around 50 kb in opossum to 370 kb in humans, but the order and the orientation of the genes within the cluster are conserved (Fig. 14.1). In mice, the γ - and δ -casein genes, which are linked within 60 kb, encode an α_{s2} -like protein, and sequence analysis suggests that the ancestral α_{s2} -casein gene duplicated at the time of radiation between rodents and Artiodactyla (George *et al.*, 1997; Rijnkels *et al.*, 1997a). A similar duplication of the α_{s2} -casein gene is also suspected to have occurred in rabbits (Dawson *et al.*, 1993). A recent duplication of β -casein was evidenced in the monotreme lineage, and the duplication of the α -casein that occurred in the eutherian lineage is absent in marsupials (Lefèvre *et al.*, 2009). Interestingly, the casein locus encompasses several other genes, expression profile of which

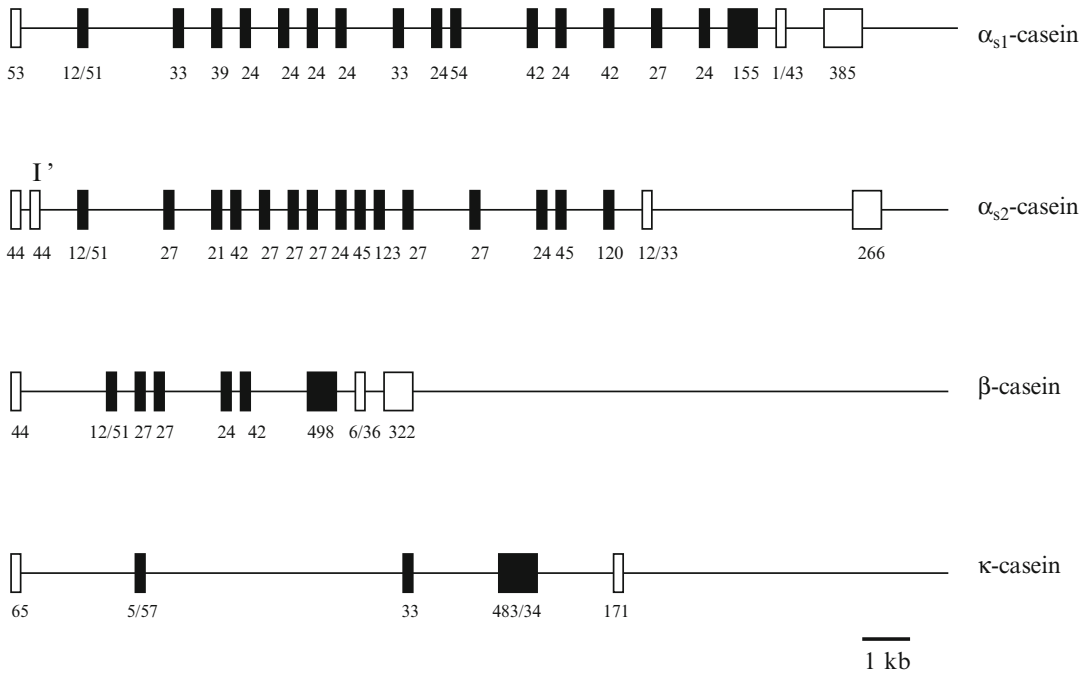


Fig. 14.2 Organization of the bovine casein-encoding genes. Exons are represented by boxes. White boxes: untranslated regions, black boxes: coding frame. Exons are not to scale and their sizes are indicated as base pairs. Two

numbers are indicated below exons that comprise both untranslated and coding sequences. Exon I' from the α_{s2} -casein gene corresponds to a partially skipped exon in the sheep species. Origins of the data are mentioned in the text

may not be restricted to the mammary gland. Inactivation of the β -casein gene (Kumar *et al.*, 1994) did not prevent expression of the remaining caseins within the locus, suggesting that they are expressed independently of each other.

The casein locus has been localized to chromosome 6 in the cow, sheep and goat (Threadgill and Womack, 1990; Hayes *et al.*, 1992; Hayes *et al.*, 1993a; Gallagher *et al.*, 1994); 5 in mice (Geissler *et al.*, 1988); 12 in rabbits (Gellin *et al.*, 1985); 4 in humans; 3 in chimpanzees (McConkey *et al.*, 1996); and 5 in opossum (Lefèvre *et al.*, 2009).

14.2.1.2 Individual Gene Structures

Internal homologies within α_{s1} - and α_{s2} -casein proteins suggested that the cognate genes evolved through intragenic duplications, a result confirmed at the DNA level by the observed duplication of intron-exon-intron stretches. The ubiquity of the major phosphorylation site and the striking homology of signal peptides of α_{s1} -, α_{s2} - and β -casein proteins indicated a possible

common origin of these calcium-sensitive casein-encoding genes. This hypothesis was further substantiated by the identification of common sequence motifs in the proximal 5'-flanking region and the similar structural organization of the first four exons. Despite its location within the casein genes cluster, the κ -casein gene appears to have no evolutionary relationship with the calcium-sensitive casein-encoding genes. It was recently hypothesized that the milk casein genes have evolved from genes involved in tooth development before the origin of mammals (Kawasaki *et al.*, 2011).

The structure of the α_{s1} -casein gene has been partially analysed in rat (Yu-Lee *et al.*, 1986) and fully determined in cow (Koczan *et al.*, 1991), goat (Leroux *et al.*, 1992) and rabbit (Jolivet *et al.*, 1992), or could be deduced from several other sequenced genomes, the same being true for the other individual milk protein genes. The transcription unit of the gene is split into 19 exons and spans around 17.5 kb in ruminants (Fig. 14.2).

The structure of the α_{s2} -casein gene has been described in cow (Groenen *et al.*, 1993). The gene transcription unit is split into 18 exons and spans 18.5 kb (Fig. 14.2). The first intron contains a non-coding exon (exon I' in Fig. 14.2) that is known to be retained in 4 % of the ovine mRNA (Boisnard *et al.*, 1991). In this species, exon VI is also partially skipped. Sequence comparisons suggest that this gene is more closely related to the β -casein gene than it is to the α_{s1} -casein gene (Groenen *et al.*, 1993).

The structure of the β -casein gene is known in mouse (Yoshimura and Oka, 1989), cow (Bonsing *et al.*, 1988), rat (Jones *et al.*, 1986), rabbit (Thépot *et al.*, 1991), goat (Roberts *et al.*, 1992), human (Hansson *et al.*, 1994) and sheep (Provot *et al.*, 1995). Its transcription unit is composed of 9 exons and spans between 8 and 10 kb according to differences between species in the length of intronic sequences (Fig. 14.2). Exon III is skipped in humans, leading to the deletion of 9 amino acids in the mature protein.

Characterisation of the κ -casein gene has been reported in cow (Alexander *et al.*, 1988; Kapelinskaia *et al.*, 1989), human (Edlund *et al.*, 1996) and rabbit (Baranyi *et al.*, 1996). The transcription unit of this gene comprises 5 exons, and its length varies from 7.5 kb in rabbit to 12.5 kb in cow (Fig. 14.2). This gene is evolutionarily related to fibrinogens (Jollès *et al.*, 1974). Indeed, a 24 bp sequence located at the 5' end of exon IV of the gene was found to be similar with the end of exon II of the γ -fibrinogen gene, suggesting that it represents an exon from the ancestral gene (Alexander *et al.*, 1988).

14.2.2 The Major Whey Protein-Encoding Genes

14.2.2.1 The β -Lactoglobulin-Encoding Gene and Pseudogenes

The structure of the β -lactoglobulin-encoding gene has been reported in sheep (Ali and Clark, 1988; Harris *et al.*, 1988), cow (Alexander *et al.*, 1993), goat (Folch *et al.*, 1994), tamar wallaby (Collet and Joseph, 1995) and horse (Lear *et al.*, 1998). The structure of this gene, with seven

exons and a transcription unit length of around 4.8 kb, was conserved during evolution (Fig. 14.3). In dogs and horses, two functional and closely linked genes are present in the genome (Halliday *et al.*, 1990; Lear *et al.*, 1998). In cats, a third functional gene is present (Halliday *et al.*, 1990; Pena *et al.*, 1999).

In ruminants, a β -lactoglobulin pseudogene has been identified (Passey and MacKinlay, 1995; Folch *et al.*, 1996), while in cow and goat a gene conversion event has occurred. The bovine and caprine pseudogenes contain seven exons, and the ancestral protein that they encode is related to the monomeric β -lactoglobulin II protein. The bovine pseudogene is located 14 kb 5' from the functional gene, in a similar orientation (Passey and MacKinley, 1995).

β -Lactoglobulin belongs to the lipocalin protein family (Flower, 1996, for review). Comparison of the structure of various lipocalin genes with that of the β -lactoglobulin gene has revealed striking similarities, confirming further their evolutionary relationship (Ali and Clark, 1988). The β -lactoglobulin gene(s) and pseudogene were assigned to chromosome 11 in cow and goat, 3 in sheep (Hayes and Petit, 1993c; Folch *et al.*, 1996) and 28 in horse (Lear *et al.*, 1998).

14.2.2.2 The α -Lactalbumin Gene and Pseudogenes

The α -lactalbumin gene has been sequenced in rat (Quasba and Safaya, 1984), cow (Vilotte *et al.*, 1987), human (Hall *et al.*, 1987), guinea pig (Laird *et al.*, 1988), goat (Vilotte *et al.*, 1991), mouse (Vilotte and Soulier, 1992), tamar wallaby (Collet and Joseph, 1995) and otariid and phocid seals (Sharp *et al.*, 2008). In eutherians, the gene is composed of four exons and its transcription unit is about 2 kb in length (Fig. 14.3). It shares the same structural organization with the lysozyme gene, corroborating the hypothesis of a common ancestor (Quasba and Safaya, 1984). The structure of the tamar wallaby α -lactalbumin gene appears different with the occurrence of a putative 5'-untranslated first exon (Collet and Joseph, 1995). In ruminants, the occurrence of related sequences, probably pseudogenes, has been reported (Soulier *et al.*, 1989; Vilotte *et al.*,

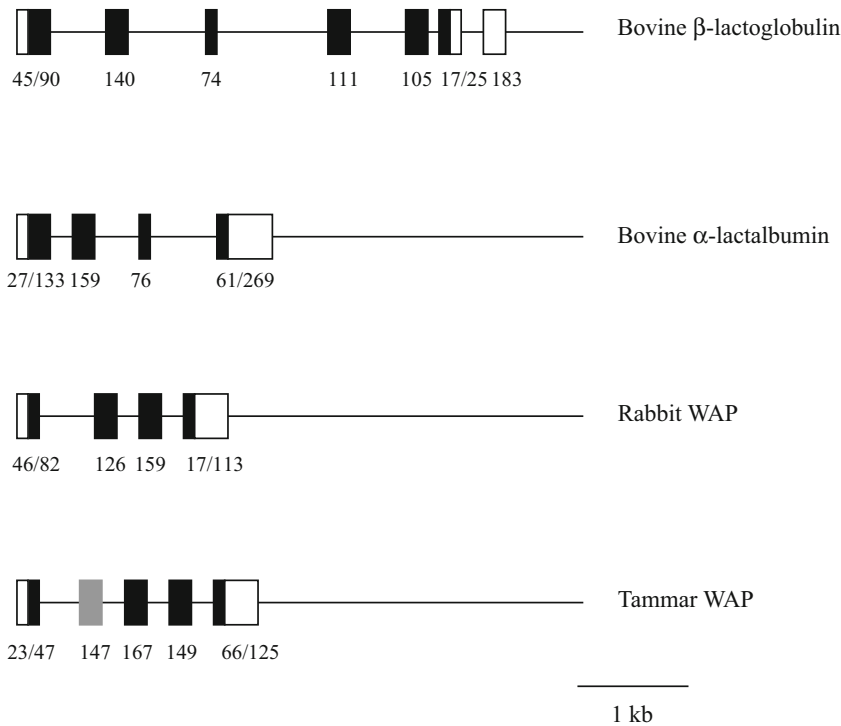


Fig. 14.3 Organization of the major whey protein-encoding genes. Exons are represented by boxes. White boxes : untranslated regions, black boxes: coding frame, grey box: exon 2 from marsupials and monotremes whey acidic pro-

tein-encoding gene. Exons are not to scale and their sizes are indicated as base pairs. Two numbers are indicated below exons that comprise both untranslated and coding sequences. Origins of the data are mentioned in the text

1991; 1993). All α -lactalbumin-related sequences are closely linked (Hayes *et al.*, 1993b; Gallagher *et al.*, 1993) and located 3' to the functional gene (quoted in Stinnakre *et al.*, 1999). The α -lactalbumin-encoding gene (and related sequences) has been assigned to human chromosome 12 (Davies *et al.*, 1987), sheep chromosome 3, bovine and goat chromosomes 5 (Hayes *et al.*, 1993b) and pig chromosome 5 (Rohrer *et al.*, 1997). In Cape fur seals, the gene appears to be transcriptionally silenced and, although comparative analysis of proximal promoter sequence revealed some differences, for which none appears to be responsible (Sharp *et al.*, 2008).

14.2.2.3 The Whey Acidic Protein-Encoding Gene

The whey acidic protein (WAP) gene was originally thought to be present only in rodents (Campbell *et al.*, 1984) but has been sequenced in

various eutherians (Thépot *et al.*, 1990; Rival-Gervier *et al.*, 2003), marsupials and monotremes (Topcic *et al.*, 2009, for review). The 2 kb transcription unit is composed of four exons in eutherians and of five exons in marsupials and monotremes (Fig. 14.3). In ruminants, the WAP gene is characterized by a nucleotide deletion at the end of exon one and a lack of detectable transcription. It thus appeared to be a pseudogene (Hajjoubi *et al.*, 2006). The functional role for WAP in milk is unknown, although it bears a similarity to a family of protease inhibitors; however, its absence in deficient mice leads to nutritional deficiencies in the offspring which appear to be unrelated to its activity as a protease inhibitor (Triplett *et al.* 2005). Both WAP and protease inhibitors of the Kunitz family are characterized by highly conserved cysteine residues located in two proteic domains (Hennighausen and Sippel, 1982). The murine WAP gene has been assigned to chromosome 11 (Gupta *et al.*, 1982).

14.3 Milk Protein-Encoding Gene Expression and Regulation

14.3.1 Tissue Specificity and Developmental Regulation

The major milk protein genes are defined as mammary-specific and developmentally regulated expressed genes. As such, they represent markers of mammary differentiation. The amount of milk protein mRNA in mammary epithelial cells increases steadily from mid-pregnancy to lactation, although at different rates according to the different genes (Harris *et al.*, 1990; Robinson *et al.*, 1995). This is due to an increase in the transcription rate of these genes as well as a stabilization of the transcripts (Guyette *et al.*, 1979). An observed asynchrony of mammary epithelial cell maturation during pregnancy (Robinson *et al.*, 1995) is paralleled by heterogeneous expression during lactation of the major milk protein genes in sheep and cattle (Molenaar *et al.*, 1992). This heterogeneous pattern of expression was not observed in lactating mouse mammary glands (Dobie *et al.* 1996). Nevertheless, a short closure of lactating murine mammary gland resulted in local perturbation of milk protein gene expression, leading to the appearance of a mosaic pattern (Faerman *et al.*, 1995). These results strongly suggest that the regulation of the expression of the major milk protein genes in mammary epithelial cell is under a complex regulation that could involve both a graded and a binary mechanism.

Over the last few years, the concept that the expression of the milk protein genes is restricted to the mammary gland has been questioned. Expression of α -lactalbumin in the rat epididymis was reported (Qasba *et al.*, 1983) and denied (Moore *et al.*, 1990; Tang, 1993). The murine α -lactalbumin gene was also reported to be expressed, alongside the β -casein gene, in the sebaceous glands during lactation (Maschio *et al.*, 1991), but this observation has not been confirmed (Persuy *et al.*, 1992; Vilotte and Soulier, 1992). RT-PCR experiments have suggested that the rat α -lactalbumin gene is also expressed at low levels in the brain of some lac-

tating animals (Fujiwara *et al.*, 1999), and casein mRNA expression has been observed in cytotoxic T-lymphocyte-derived cell lines (Grusby *et al.*, 1990). Occurrence of casein-like immunoreactive substances in diverse organs, including the thymus, was reported in the rat (Onoda and Inano, 1997). More recently, promiscuous milk protein gene expression was observed in medullary thymic epithelial cells, probably in relation with the development of central T cell tolerance (Derbinski *et al.*, 2008). It is of interest to note that many of the tissues where ectopic expression is observed contain some of the transcription factors required for mammary expression of the milk protein genes, for example, in T cells, interleukin-2 activates STAT5 (Gilmour *et al.*, 1995).

14.3.2 Hormonal Regulation and Identification of *cis*-Regulatory Elements

Expression of the major milk protein-encoding genes is under a complex multi-hormonal regulation, resulting from the interplay of steroid and polypeptide hormones. In addition, local growth factors and cell-cell and cell-substratum interactions are also involved. Since many reviews have already focused on this topic, including the previous edition of this chapter (Vilotte *et al.*, 2002), we will only give a brief survey here.

Schematically, lactogenic hormones, such as insulin, prolactin and glucocorticoids, activate the transcription of the major milk protein genes whereas other hormones, such as progesterone, inhibit this activation in the early stages of pregnancy to favour cell proliferation over cell differentiation. It should be noted that in many systems it is difficult to separate direct induction of milk protein transcription from indirect differentiation-related events. As already mentioned, transcription activation of the major milk protein genes is not concomitant during pregnancy, perhaps due to the presence of various hormonal micro-environments and/or different responses to this environment displayed by different milk protein genes. For example, expression of calcium-sensitive casein genes that are activated at

mid-pregnancy relies on prolactin and is increased by the synergetic action of glucocorticoids (Kabotyanski *et al.*, 2006, 2009), while the WAP promoter, a gene expressed late in pregnancy, is reciprocally regulated by these two hormones. In addition, whereas the glucocorticoid induction of the WAP gene expression is rapid, its action on the β -casein gene occurs only with a significant time-lag and requires *de novo* protein synthesis. Expression of the β -lactoglobulin gene, although displaying a similar temporal expression profile to the caseins, appears to be less dependent on lactogenic hormones. Finally, the α -lactalbumin gene, although displaying a temporal expression profile similar to WAP, is induced by prolactin in the presence of low concentrations of glucocorticoids whereas high concentrations of glucocorticoids inhibit its expression, at least in eutherians (Funder, 1989). Induction of the α -lactalbumin gene in the absence of prolactin could be observed in the pregnant murine mammary explants in the presence of insulin and cortisol (Warner *et al.*, 1993), while in marsupials, α -lactalbumin gene expression depends only on prolactin (Collet *et al.*, 1990). The mechanistic rationale for these intriguing differences has not been identified. Finally, it was recently shown that beside transcriptional regulation and transcript stabilization, lactogenic hormones are also involved in the translational regulation of milk protein synthesis (Rhoads and Grudzien-Nogalska, 2007, for review).

Regulation of mammary gene expression is also controlled by the epithelial cell basement membrane. For example, laminin can induce expression of α -lactalbumin, α_{s1} -casein and β -casein by 160-fold (Aggeler *et al.*, 1988; Blum *et al.*, 1989). The differences observed between milk protein genes with regard to hormonal induction are also evident in their varying requirement for a basement membrane. Transcriptional control of the β -casein promoter appears less dependent on the three-dimensional structure of the mammary epithelial cells than does the WAP promoter, although both of them are sensitive to extracellular-matrix (ECM) components (Lin *et al.*, 1995). At least for the

β -lactoglobulin gene, regulation of expression by the ECM occurs through activation of STAT5 (Streuli *et al.*, 1995), and this may occur through an ECM-dependent modulation of protein-tyrosine phosphatase activity (Edwards *et al.*, 1998). Cell-substratum components as well as glucocorticoids can, at least for the casein genes, also act at the post-transcriptional level (Eisenstein and Rosen, 1988).

Beside these differences, milk protein genes share specific hormonal responses. For example, in most species, progesterone inhibits milk protein gene expression. Although the exact mechanism is still unclear, it has been reported that progesterone might repress expression of the long form of the prolactin receptor mRNA in the mammary gland (Mizoguchi *et al.*, 1997) and/or directly repress the prolactin/STAT5-mediated transcription at the milk protein gene promoter level (Buser *et al.*, 2007).

Transfection and transgenic studies have revealed that the promoters of most of the major milk protein-encoding genes are responsive to lactogenic hormones sufficiently to target mammary-specific expression of reporter genes in transgenic animals. However, these promoters cannot sustain full expression on their own (compare Webster *et al.*, 1995 with Whitelaw *et al.*, 1992). Indeed, intragenic sequences of some of the major milk protein-encoding genes were shown to be able to contribute to their hormonal regulation (Lee *et al.*, 1989), and important regulatory elements have been identified within the introns (Kang *et al.*, 1998, Kolb, 2003) and/or in the 3' untranslated region (UTR) and flanking regions (Dale *et al.*, 1992). Furthermore, and as already mentioned, milk protein genes are also regulated at a post-transcriptional level.

Sequence comparison of a particular gene between several species or from different milk protein genes has led to the identification of conserved DNA motifs that were suspected to be involved in the control of the gene transcription. A classical example is the high conservation of sequence elements in the proximal 5'-flanking region (-200/+1) of the calcium-sensitive casein genes (Rosen, 1987; Kolb, 2002). These early identified elements were subsequently found to

be recognized by regulatory nuclear factors. Consensus sequences recognized by effectors known to be involved in lactogenesis were also identified within the major milk protein gene promoter sequences by computer searches. However, evidence for the presence of a *cis*-regulatory element within a DNA fragment came from transfection experiments in cell cultures, transgenic studies, DNase I protection, footprinting and/or gel shift assays. Site-directed mutagenesis of the identified binding sites and observations, either in cell culture or in transgenics, of the consequences of such mutations on the promoter transcriptional regulation was sometimes performed to further define the functional role of these elements.

14.3.3 Transcriptional Control of Milk Protein Genes

Binding sites for several transcription factors have been identified within the promoters of most of the major milk protein-encoding genes, such as binding sites for OCT-1, NF-1, C/EBP, STAT5, GR, Ets-1 and YY1 (see Rosen *et al.*, 1996, for review). Other DNA elements have been shown to interact with yet unidentified effectors, such as the negative regulatory elements of the WAP promoter (Kolb *et al.*, 1994) and of the β -casein promoter (Lee and Oka, 1992; Altioik and Groner, 1993, 1994). Most of these sequences appear to be clustered within short DNA fragments of several hundred base pairs in length that encompassed both positive and negative regulatory elements. Such composite response elements have been identified in the proximal 5'-flanking regions of the β -lactoglobulin gene (region -406/+1; Watson *et al.*, 1991), of the calcium-sensitive casein genes (region -200/+1; Rosen *et al.*, 1986; 1998 for review), in more distal regions of the bovine (BCE-1 element: region -1613/-1562; Schmidhauser *et al.*, 1992, Myers *et al.*, 1998) and human (region -4700/-4550; Winklehner-Jennewein *et al.*, 1998) β -casein genes, of the rabbit α_{s1} -casein gene (region -3442/-3118; Pierre *et al.*, 1994) and of the rat WAP gene (region -949/-720; Li and Rosen

1994a, 1995; Raught *et al.*, 1995). A cooperation of distal and proximal promoter elements is required to achieve both maximum expression and maximum hormone responsiveness of the murine β -casein gene in mouse HC11 cells (Robinson and Kolbs, 2009). Thus, it appears that the transcriptional regulation of the major milk protein genes is under a combinatorial control with the binding of multiprotein complexes that can either repress or activate gene expression (see Wolberger, 1998, for review).

Differences in the composition of these composite regulatory elements may explain the observed hormonal differences in the transcriptional developmental regulation between the various major milk protein genes. In the WAP gene, for example, an Ets-1 binding site located at -110 appears to be important for the stage-specific transcriptional activation of the gene but not for its stable expression during lactation (McKnight *et al.*, 1995). Activation of promoters by transcription factors can be mediated by the relief of the binding of transcription repressors through both the competitive binding of these activators and the hormonal regulation of the expression or activation of these factors (Schmitt-Ney *et al.*, 1991). Thus, some negative binding factors appear to be present in the mammary gland only during pregnancy but not during lactation (Lee and Oka, 1992). Some of them mediate the inhibitory action of progesterone. Similarly, expression of C/EBP β protein isoforms that are essential both for mammatogenesis and lactogenesis (Robinson *et al.*, 1998; Seagroves *et al.*, 1998) is regulated during pregnancy and lactation. The ratio between LIP, a dominant-negative transcriptional repressor, and LAP, which is an activator of transcription, is high during the pregnancy stage and decreases during lactation due, in part, to the inhibition of LIP expression by glucocorticoids (Raught *et al.*, 1995). Expression of C/EBP α is also increased during lactation, possibly following stimulation by some ECM components (Raught *et al.*, 1995). The action of another factor, the transcription factor YY1, that represses expression of the β -casein promoter by binding to it at position -120/-110 is counteracted by its replacement from its binding site by the

prolactin-activated STAT5 protein (see below), which in turn positively regulates the promoter activity (Meier and Groner, 1994). A more surprising example of regulation *via* binding of negative regulatory factors is given by the two proteins that bind the upper strand of the β -casein promoter at position -221/-170 during pregnancy and involution (Altiok and Groner, 1994). During lactation, a molecule inhibits the binding of these factors to the gene promoter, and it is suspected that this molecule could be the β -casein mRNA itself that possesses high-affinity binding sites for the two proteins in its 5' UTR (Altiok and Groner, 1994).

Much has been discovered about how milk protein genes are regulated, and the involvement of many transcription factors has been described. Notwithstanding all this information, the identification of STAT5 as the end point of prolactin signalling in the mammary gland heralded a new era in our understanding of mammary gene regulation. All the more so, since the STAT proteins are central to all cytokine responses (Heim, 1999). Much of the work on STAT proteins has been pioneered by studies involving mammary genes (Schmitt-Ney *et al.*, 1991; Watson *et al.*, 1991).

14.3.4 Prolactin Signal Transduction

In the mammary gland, prolactin induction results in the expression of the milk protein genes. This occurs through a rapid but transient signalling transduction pathway (Heim, 1999). First, prolactin-induced dimerisation of its receptor causes *trans*-phosphorylation of the kinase which is constitutively associated with the cytoplasmic domain of the receptor. The kinase is called JAK2 (for janus kinase 2). The activated JAK2 phosphorylates the receptor creating a docking site for STAT5 through its SH2 domain. Subsequent phosphorylation and dimerisation of STAT5 result in its translocation to the nucleus where it binds to GAS (γ -interferon activation sequences) elements in target genes, for example, β -lactoglobulin. Many other proteins are associated with this pathway, for example,

MAPK and SOCS, resulting in a logistical problem for the cell to manage if it is to maintain tight regulation of the signal. It does this by balancing the activation signal with the generation of factors that inhibit the signal (Starr and Hilton, 1999; Tomic *et al.*, 1999).

STAT5 is an important positive transcription factor in the transcriptional regulation of milk protein genes (see Barash, 2006, for review). It is involved in the transduction of the prolactin signal. Functional STAT5 binding sites have been identified in the promoter region of almost all major milk protein-encoding genes, with the potential exception of the κ -casein-encoding gene (Adachi *et al.*, 1996). Within calcium-sensitive casein and β -lactoglobulin composite elements, the occurrence of multiple STAT5 binding sites is observed. These STAT5 binding sites were shown to be essential to confer prolactin transcriptional stimulation to the linked promoter (Schmitt-Ney *et al.*, 1991; Demmer *et al.*, 1995; Jolivet *et al.*, 1996; Soulier *et al.*, 1999). Furthermore, several experiments suggest that STAT5 effects are limited to the modulation of expression level, but are not involved in determining the tissue specificity of expression. For example, mutation of one or several of the STAT5 binding sites within the β -lactoglobulin promoter did not affect the tissue-specific expression of this gene in transgenic mice (Burdon *et al.*, 1994).

The STAT5 transcription factor actually consists of two proteins, STAT5a and STAT5b. These highly similar proteins are encoded by two genes, with differences in their binding affinities due to a single amino acid substitution (Boucheron *et al.*, 1998). The role of these proteins has been studied using gene knockout approaches, with STAT5a emerging as the major factor required for milk protein gene expression. STAT5a-deficient mice exhibit defective mammary gland development (Liu *et al.*, 1997). As one might expect, given the different response of the various milk protein genes to hormones, STAT5a knockout mice express the milk protein genes to different levels. Essentially normal levels of β -casein and α -lactalbumin mRNA levels are detected, with only WAP mRNA levels showing

a reduction (Liu *et al.*, 1997). Indeed, there is no correlation between the expression of the STAT5a and the β -casein genes (Kazansky *et al.*, 1995). Studies with STAT5b knockout mice indicate that this protein is associated with growth hormone effects (Udy *et al.*, 1997).

Altogether, these observations suggested that STAT5 may facilitate the interaction of other transcription factors to allow the transcriptional activation of the promoter. Indeed, functional interaction between STAT5 and the glucocorticoid receptor was reported, and this molecular complex was demonstrated to cooperate in the induction of the β -casein promoter, independently of the DNA-binding function of the glucocorticoid receptor (Stöcklin *et al.*, 1996, 1997; Lechner *et al.*, 1997).

Better understanding of the nature and structural arrangement of the *cis*-regulatory elements involved in the control of the transcription of the major milk protein genes has given an insight into the differential developmental regulation of their expression. However, many questions remain. Why is expression of WAP in preference to β -casein affected in the STAT5a-null mice given that the reciprocal requirement for prolactin is observed? If it is not STAT5, and it appears not to be, what confers mammary specificity to milk protein gene expression? How do the various transcription factors involved interact with each other to generate a production transcription complex? How does this transcription complex interact with the underlying nucleosomes?

14.3.5 Milk Protein Gene Chromatin Domains

It is now generally accepted that chromatin, or its epigenetic regulation, plays a central role in the regulation of gene expression (Rijnkels *et al.*, 2010). Studies on the ovine β -lactoglobulin gene brought indirect, although compelling, evidence that chromatin reorganization is important for milk protein gene expression. Concomitant to the increase in β -lactoglobulin gene expression during pregnancy, due to activation of STAT5, changes in DNase I hypersensitivity in the β -lactoglobulin

gene were reported to occur (Whitelaw and Webster, 1998). This implies a reorganization of the chromatin in response to the interaction of transcription factors. Furthermore, this assay has detected species-specific differences in this reorganization of the chromatin (Pena *et al.*, 1998). These differences are important clues to understand the various levels of expression of the same gene between species or between alleles (Whitelaw, 2000). Similar studies have shown that chromatin reorganization events also occur during the induction of the expression of the rat WAP gene (Li and Rosen, 1994b). However, the clearest evidence yet describing a role for chromatin structure in the regulation of milk protein gene expression comes from the analysis of the β -casein BCE-1 element. This element is not activated in transient transfections, in which true chromatin is not formed, and is responsive to the state of acetylation of the histones (Myers *et al.*, 1998).

The transcription domains of the major milk protein genes are poorly defined. As yet, they have generally been defined through functional analyses only. Transgenic studies have revealed that while the α_{s1} - and the β -casein genes from various species can be expressed at relatively high levels in mice, only weak expression of the α_{s2} - and the κ -casein transgenes was observed. It is thus hypothesized that a mammary-specific locus control region (LCR) might control the expression of the casein locus and that this putative element is located close to the α_{s1} - and the β -casein loci. So far, however, no direct evidence for the existence of such an element has been provided. Furthermore, as the casein gene clusters of some species contain several genes which are not expressed tissue specifically in the mammary gland (Fig. 14.1), the influence of a putative LCR would have to be selective for the casein genes.

A consequence of chromatin gene domains is that boundaries to these domains must exist. There are several documented examples (see Geyer, 1997, for review), but again, as yet, none has been conclusively identified for a milk protein gene. However, some predictions are possible using genome comparison. The boundaries of the α -lactalbumin gene are not known, but those of the related lysozyme gene are well defined (Phi-Van

and Strätling, 1988). It is likely that the α -lactalbumin gene contains similar elements, probably in a similar location. This hypothesis is supported by the observed position-independent and copy number-related expression of a human 250 kb YAC and of a 160 kb goat BAC- α -lactalbumin transgene (Fujiwara *et al.*, 1997; Stinnakre *et al.*, 1999).

At present, the transcription domain of the β -lactoglobulin gene might be one of the best studied within milk protein genes. Several DNase I-hypersensitive sites that reflect its expression status have been located within the promoter, intronic and 3'-flanking regions (Whitelaw and Webster, 1998). These sites spatially reflect the limit of the chromatin domain, as defined by nuclease sensitivity. The 5' limit of this domain resides very close to the promoter. In addition, the proximal 3'-flanking β -lactoglobulin sequences can interact with the nuclear matrix *in vitro*. This suggests that the β -lactoglobulin gene resides in a very small chromatin domain (Whitelaw, unpublished results). Sequences that interact with the nuclear matrix, usually AT-rich in nature, may be involved in regulating chromatin structure thereby facilitating gene expression. Although several studies have addressed the ability of such sequences to enhance milk protein gene expression, a clear picture has not yet emerged (e.g., Attal *et al.*, 1995; McKnight *et al.*, 1996). Another well-studied locus is that encompassing the WAP gene, the DNA of which adopts different chromatin loop structures according to the studied tissue and developmental stage that allow the differential expression of the genes it contains (Montazer-Torbati *et al.*, 2008) and regulation from distant enhancers and/or repressors (Saidi *et al.*, 2007).

14.3.6 Milk Protein mRNAs

The structure of milk protein mRNAs has been investigated in numerous species (see Mercier and Vilotte, 1993, for review). In lactation, these RNAs account for up to 60 %–80 % of the total RNA present in mammary epithelial cells. The calcium-sensitive casein mRNAs are characterized by a better interspecies conservation of the UTRs and of the sequence encoding the signal peptide compared to the mature protein-coding

frame. Since part of the regulation of milk protein gene regulation is exerted at a post-transcriptional level (see Aggeler *et al.*, 1988; Blum *et al.*, 1989; Golden and Rillema, 1995, for examples), conservation of the UTR might reflect their importance for the mRNA processing. In many cases, the rate of translation is influenced by the 5' UTR sequence and its secondary structure. Sequences located in the 3' UTR are also known to interact with proteins to either stabilize or degrade the RNA, and/or alter mRNA translation efficiency, and to be potential targets for miRNA. Involvement of miRNA in the biology of the mammary gland is still poorly documented. However, recent studies suggested their implication during normal mammary gland development and differentiation (Gu *et al.*, 2007; Wang and Li, 2007; Avril-Sassen *et al.*, 2009; Sdassi *et al.*, 2009; Tanaka *et al.*, 2009; Ucar *et al.*, 2010). A direct role for miRNA in the synthesis of a milk protein was recently evidenced with the identification of a conserved region in the 3' UTR of the lactoferrin gene targeted by miR-214 (Liao *et al.*, 2010).

As already mentioned, total or partial exon skipping during the splicing of the pre-mRNA is responsible for the differences observed between casein variants and between homologous proteins from different species. In this section, we will just describe variations that affect the overall level of gene expression. Deletion or transition of a single nucleotide within coding exons of a caprine α_{s1} -casein allele and of two β -casein alleles creates nonsense codons (Leroux *et al.*, 1992; Persuy *et al.*, 1996; Rando *et al.*, 1996). These alleles are characterized by a much lower level of mRNAs compared to other alleles and, for some of them, with multiple exon-skipping events. This phenomenon has been observed for several other genes where nonsense codons have been found to be associated with mRNA decay and/or exon skipping (see Valentine, 1998; Hentze and Kulozik, 1999, for reviews). Goat and bovine α_{s1} -casein alleles, also associated with reduced amount of mRNA and milk protein, were found to contain a truncated inserted LINE element in the 3'UTR (Perez *et al.*, 1994; Rando *et al.*, 1998). Insertion of these elements is supposed to reduce mRNA stability.

14.4 Milk Protein Synthesis and Secretion

Secretion of milk protein is obviously of interest due to its physiological and economic importance but also from the point of view of the study of high-efficiency secretory pathways. The trafficking and processing events leading to the secretion of milk proteins are known in general outline, but relatively little is established of the molecular cell biology of milk protein transport in the secretory pathway of mammary epithelial cells.

14.4.1 Morphological Organization of the Mammary Secretory Epithelium

Functional differentiation of the mammary gland is linked to the development of its epithelial tissues. From the onset of pregnancy, the duct cells enter a proliferation and differentiation period leading to the development of a highly branched ductal tree which fills the entire mammary fat pad. Alveolar structures, or acini, develop at the ends of the side branches, and terminal differentiation of the alveolar mammary epithelial cells is completed at the end of gestation with the start of milk secretion at parturition. Functional acini are embedded in a stroma composed of connective and adipose tissues, fibroblasts, plasma cells, blood vessels and nerve terminals. These morphological aspects are well documented at <http://mammary.nih.gov/>

The acini consist of a single layer of cuboidal mammary epithelial secretory cells sealed together at the luminal border by tight junctions so that passage of molecules from the interstitial space to the lumen of the acini by a paracellular route is restricted during lactation, confining such molecular movements to the cellular trans-cytotic pathway. Progenitor cells are believed to reside below the monolayer of luminal cells. Contractile myoepithelial cells that have long spidery processes embrace the alveoli secretory cells. They participate in milk ejection by squirting milk out of the acini lumen into the ducts. Finally, these

cells that constitute the acini are separated from the interstitial space by a basement membrane like in any epithelial tissue.

14.4.2 The Functional Compartmentalisation of the Biosynthetic-Secretory Pathway of Mammary Epithelial Cells

During lactation, mammary epithelial cells are highly polarized and display features typical of cells specialized for secretion (Bargmann and Knoop, 1959; Wooding, 1977; Pitelka and Hamamoto, 1983; illustrated in Figs. 14.4 and 14.5). Their cytoplasm contains an extended network of numerous parallel lamellar cisternae and branching tubules decorated with electron-dense ribosomal particles: the rough endoplasmic reticulum (ER). As described in other polarized epithelial cells, the Golgi apparatus is typically located in the peri- and supranuclear region of the cell, close to the centrosome. The Golgi cisternae are well developed, more or less distended, often containing electron-dense particles and filamentous materials. Of note, unusually large vesicles are observed on the *trans* side of the Golgi apparatus. It is not clear whether these dilated structures constitute the *trans*-Golgi network (TGN) or represent newly formed secretory vesicles *en route* to the apical plasma membrane (APM). In addition, numerous smaller vesicles, some of them coated with the typical spike structure characteristic of clathrin, are associated with the *trans* side of the Golgi. Secretory vesicles that have pinched off from the TGN contain filamentous structures and casein micelles similar to those found in milk, in an electron-lucent fluid.

14.4.3 Intracellular Transport and Co- and Post-translational Modifications of Milk Proteins

In mammary epithelial cells, transport of newly synthesized proteins destined for secretion proceeds according to the general biosynthetic-secre-

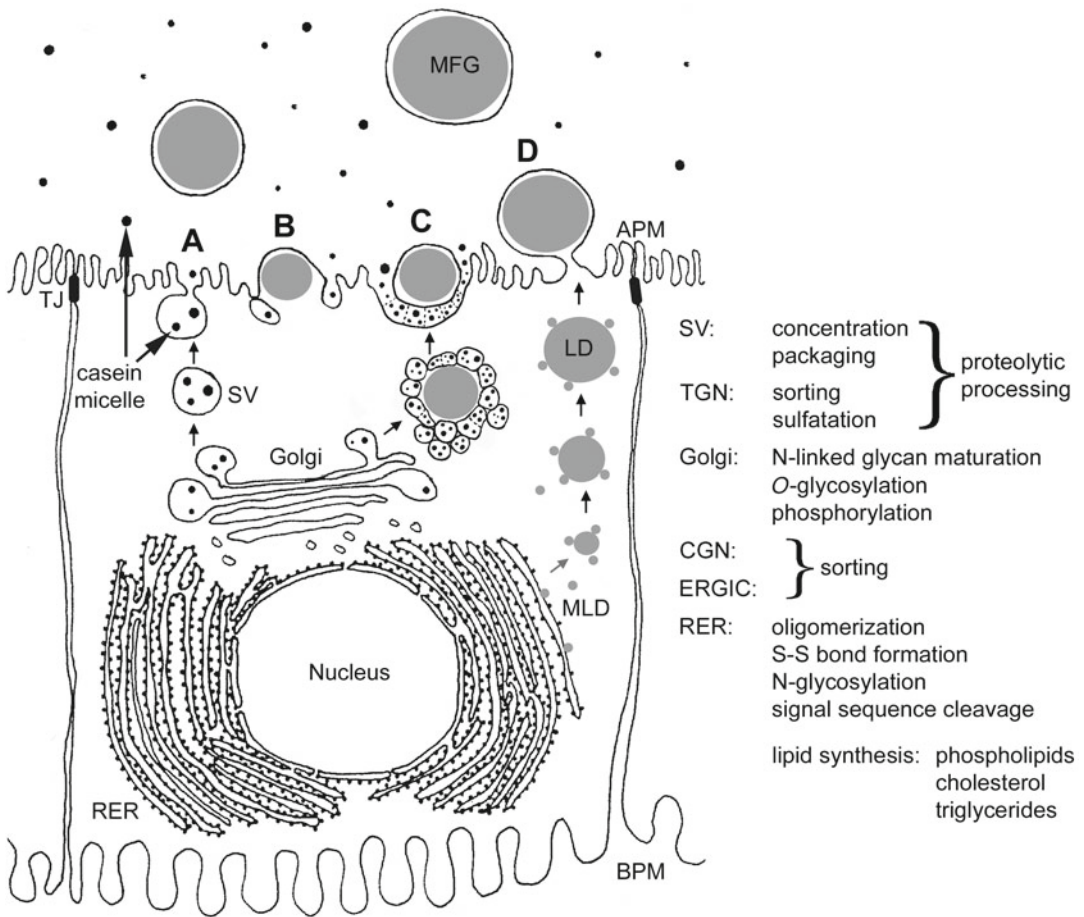


Fig. 14.4 The intracellular compartments of the mammary epithelial cell involved in the biosynthesis and secretion of milk proteins and lipids. Milk proteins undergo various co- and post-translational modifications during their translocation into the lumen of the rough endoplasmic reticulum (RER) and further transport to the apical cell surface, *via* the Golgi apparatus. Casein self-association starts in the lumen of the RER and proceeds into long loose linear aggregates in the *trans*-Golgi compartments and early secretory vesicles (SV). They further self-associate before release, and structures with the characteristic morphological aspect of casein micelles are found in distal SV (see Fig 14.5). Casein-containing SV reach and fuse with the apical plasma membrane (APM) by exocytosis (pathway A). Microlipid droplets (MLDs) emerge from the RER and may fuse with each other and with larger cytoplasmic lipid droplets (CLDs) as they are trans-

ported to the apex of the mammary epithelial cells. CLDs and caseins secretion may be partly (pathway B) or significantly (pathway C) coupled (dotted lines indicate that membrane of the casein vesicles has fused together) since the budding of the CLDs requires considerable amounts of APM. Pathway B may only involve heterotypic fusion between the casein vesicles and the APM while pathway C may imply both homotypic between SVs and heterotypic fusion of SVs with the APM. Alternatively, CLDs may be secreted independently of casein-containing SV (pathway D), and some MLDs may also be directly secreted at the apical side of the MECs (not shown). Basal plasma membrane (BPM), *cis*-Golgi network (CGN), ER-Golgi intermediate compartment (ERGIC), lipid droplet (LD), milk fat globule (MFG), tight junction (TJ), *trans*-Golgi network (TGN). Redrawn from Chat *et al.* (2011) (Courtesy of S. Truchet)

tory pathway (Palade, 1975). Following a 3-min pulse labelling with a radioactive amino acid, newly synthesized proteins are detectable in the ER 5 min after the beginning of the pulse, concentrated in the

Golgi region after 15 min, accumulated in apical secretory vesicles after 45 min and are predominantly located in the lumen of the acini after 60 min (Seddiki and Ollivier-Bousquet, 1991).

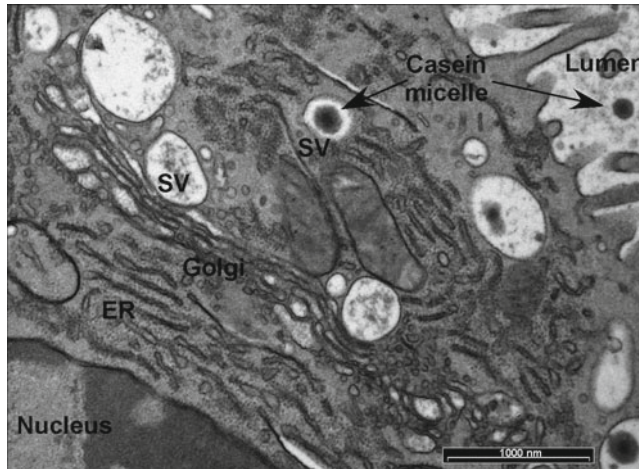


Fig. 14.5 Transmission electron micrograph of part of a mouse mammary epithelial cell during lactation. The major compartments of the secretory pathway are illustrated. Filamentous material is found in dilated Golgi cisternae and in secretory vesicles (SV) close to the

trans-Golgi, whereas more condensed protein aggregates and typical casein micelles are present in more distal SV and in the lumen of the acini. ER, endoplasmic reticulum (Courtesy of S. Chat and C. Longin)

14.4.3.1 Translocation into and Transport from the Endoplasmic Reticulum

Milk proteins are of two types: transmembrane proteins and water-soluble proteins. An example of milk transmembrane protein is butyrophilin which is first targeted to the plasma membrane from which it is released into milk as part of the milk fat globule (MFG) membrane. The vast majority of milk proteins, however, are water-soluble proteins, for example, the caseins. Entry into the ER requires a signal sequence (Blobel and Dobberstein, 1975). Translocation of milk protein polypeptide chains into the ER lumen and subsequent cleavage of their signal sequence involve classical mechanisms (Schatz and Dobberstein, 1996).

Once translocated into the ER lumen, proteins are in an oxidizing environment which promotes the formation of disulfide bonds between cysteine residues. Most proteins synthesized in the rough ER are glycoproteins. They are modified by a common oligosaccharide on target asparagines residues (*N*-glycosylation). *N*-linked oligosaccharides are added co-translationally and serve as tags to monitor the state of protein folding. The proper folding of proteins that are made in the ER

is assisted by proteins with chaperone activities, including BiP/GRP78, Erp77, calnexine and calreticulin (Pelham, 1989). These ER-resident proteins bind to misfolded newly synthesized proteins and retain them in the ER until they have achieved their properly folded or oligomeric state. Correct protein folding or oligomerisation is prerequisite for protein export from the ER and transport to the Golgi complex. This process has been named “quality control” (Hammond and Hellenius, 1995). Accumulation of improperly folded proteins in the ER causes a stress which triggers a coordinated adaptive programme called the unfolded protein response (UPR).

Elucidation of the secondary structure of α -lactalbumin and β -lactoglobulin has revealed the presence of four and two disulfide bridges, respectively (Brew *et al.*, 1970; Papiz *et al.*, 1986). As to WAP, it contains numerous cysteine residues which form multiple intramolecular disulfide bonds (Hennighausen and Sippel, 1982; Devinoy *et al.*, 1988). Moreover, native WAP is dimeric (Baranyi *et al.*, 1995). Analysis of the primary structure of κ -casein reveals the very high degree of conservation of at least one cysteine residue in the N-terminal domain of the protein (Bouguyon *et al.*, 2006). Dimers of

κ -casein were found in all milk studied, and interchain disulfide bridges were also found for any casein possessing a cysteine residue (Bouguyon *et al.*, 2006), for example, in mouse and rat which express four or five cysteine-containing caseins, respectively. As expected, disulphide bond formation between casein molecules was demonstrated to occur within the ER lumen (Le Parc *et al.*, 2010). Finally, *N*-glycosylation of rat α -lactalbumin has been detected in mammary microsomal membranes (Lingappa *et al.*, 1978). Although *N*-glycosylated forms of α -lactalbumin have been observed in several species, only rat α -lactalbumin is efficiently glycosylated (Prasad *et al.*, 1979; Chanut, 2006).

Caseins are not *N*-glycosylated and were shown to lack appreciable amount of regular secondary structure. β - and κ -caseins might possess premolten or molten globule conformations whereas α_{s1} - and α_{s2} -caseins are intrinsically unstructured proteins (Farrell *et al.*, 2006; see Chap. 5) or natively unfolded proteins (Holt and Sawyer, 1993). The characteristic structural feature of natively unfolded proteins is a combination of low mean hydrophobicity and relatively high proportion of charged residues at physiological pH (Uversky *et al.*, 2000). Proteins with such an open structure possess a peculiar aggregative behaviour and are prone to interact with their specific ligand *in vivo*. The caseins, however, do not fulfil these two criteria since they present relatively high hydrophobicities.

To date, the question whether caseins are subjected to the ER quality control machinery has not been directly addressed. Moreover, whether or not soluble luminal cargo proteins need to be concentrated and/or require intrinsic sorting information for loading into COPII-coated transport carriers at the ER exit sites to move to the Golgi apparatus is still controversial (for review see Lee *et al.*, 2004). Some secretory proteins seem to enter a transport carrier without positive selection by a signal-independent mechanism known as “bulk flow” (Rothman and Wieland, 1996). Finally, as stated above, subunit oligomerisation is required for export of multimeric proteins from the ER (Copeland *et al.*, 1986; Kim and Arvan, 1991). Whether interaction between

at least some of the caseins in the ER lumen is a prerequisite for their forward transport to the Golgi apparatus is not clear. However, investigation of the impact of the polymorphism at the α_{s1} -casein locus on goat milk secretion has shown that, in the absence of α_{s1} -casein, other caseins accumulate in the ER (Chanut *et al.*, 1999). The efficiency of casein transport from the ER to the Golgi apparatus was strongly affected in this context. Data suggested that interaction of caseins in a yet-to-be-identified structure including α_{s1} -casein is required for efficient transport of these proteins to the Golgi apparatus. More recently, the existence of a membrane-associated form of α_{s1} -casein in the ER and more distal compartments of the secretory pathway of mammary epithelial cells has been reported (Le Parc *et al.*, 2010), further suggesting a key role of α_{s1} -casein in casein transport in the biosynthetic pathway.

From the above considerations, it is now obvious that first interactions between the caseins take place in the ER and that the exit of casein from this compartment is a key step in casein micelle biogenesis and casein transport in the secretory pathway.

14.4.3.2 Transport Through the Golgi Apparatus

Protein transport through the Golgi apparatus may occur according to the vesicular transport model (stable Golgi cisternae) or the cisternal maturation model (Glick and Malhotra, 1998). Many lines of evidence now support this later model in which the Golgi cisternae themselves move through the Golgi stack. The observation that electron-dense structures, most likely casein aggregates, are detectable in Golgi cisternae of lactating mammary epithelial cells but are excluded from Golgi-associated vesicles is in agreement with this cisternal maturation model (Clermont *et al.*, 1993).

Golgi enzymes carry out protein modifications that include glycosylation, phosphorylation, sulphation and proteolytic processing (Fig. 14.4). In mammary epithelial cells, glycosyltransferases are notably involved in the *O*-glycosylation of κ -casein. Moreover, galactosyltransferase, with the help of α -lactalbumin, is also responsible for

the synthesis of lactose (Ebner and Brodbeck, 1968). Galactosyltransferase activity has been detected within the Golgi apparatus but also in secretory vesicles and milk (Witsell *et al.*, 1990; Boisgard and Chanut, 2000). The synthesis of lactose in the *trans*-most cisternae of the Golgi apparatus, and most likely in secretory vesicles, surely explains the swollen aspect of these organelles in mammary epithelial cells. The striking reduction of the volume of casein-containing secretory vesicles in α -lactalbumin-deficient mice is in agreement with this hypothesis (Stinnakre *et al.*, 1994). Phosphorylation of the calcium-sensitive caseins on serine clusters allows calcium phosphate binding and further interactions between caseins. Like in other cell systems, the kinases that phosphorylate the caseins are located within the Golgi apparatus (Bingham and Farrell, 1974; West and Clegg, 1984). Notably, the phosphorylation of β -casein seems delayed compared to that of α_{s1} -casein (Turner *et al.*, 1993; Boisgard and Chanut, 2000; P  choux *et al.*, 2005). This suggests that strong interaction of β -casein with casein polymers might be postponed until it is trafficked to *trans*-Golgi cisternae (see below). Protein sulphation is a ubiquitous TGN-specific post-translational modification. Beside sulphated proteoglycans (Boisgard *et al.*, 1999), the sulphation of both α -lactalbumin and κ -casein from rat has been described (Chanut, 2006). On the other hand, although no direct evidence for the cleavage of milk proteins by endoproteases has been reported, furin has been shown to be relatively abundant in Golgi-derived clathrin-coated vesicles from lactating rabbit mammary epithelial cells (Pauloin *et al.*, 1999).

14.4.3.3 Transport from the *trans*-Golgi Network and Secretory Vesicle Exocytosis

Secretory proteins are segregated, highly concentrated and packaged into appropriate transport vesicles in the TGN. Sorting and concentration are believed to involve the selective aggregation of the secretory proteins in the ionic environment of the TGN (Chanut and Huttner, 1991), as well as retrieval of excess membrane and luminal con-

tent present in newly formed secretory vesicles in clathrin-coated vesicles (Tooze, 1998).

Pre-micellar aggregates have been observed by electron microscopy in the lumen of the Golgi cisternae (Clermont *et al.*, 1993). Consistent with the presence of a high calcium concentration in the *trans*-most Golgi cisternae (Neville and Watters, 1983), there is a drastic rearrangement of the micellar structure during the formation of secretory vesicles at the TGN and their transport to the APM for exocytosis. In newly formed transport vesicles, caseins are concentrated in the form of long loose linear aggregates (Mather and Keenan, 1983; Clermont *et al.*, 1993). These progressively self-associate and become bigger and denser, and structures with the characteristic honeycomb texture of casein micelles from milk are found in distal secretory vesicles (see Fig. 14.5). The mean size of casein micelles varies widely across species, and the relative proportion of κ -casein was demonstrated to be a modulator of micelle size (Gutierrez-Adan *et al.*, 1996). Noteworthy, casein micelles, or at least big aggregates, still form in mammary epithelial cells from α_{s1} -, β - or κ -casein-deficient animals (Kumar *et al.*, 1994; Chanut *et al.*, 1999; Shekar *et al.*, 2006). Interactions between the various caseins and minerals during micelle biogenesis in the secretory pathway might therefore involve rather general physico-chemical and biochemical characteristics of these components. However, these characteristics are specific enough to avoid incorporation of whey proteins in the micelles. On the other hand, casein-containing secretory vesicles might undergo maturation before exocytosis (Pauloin *et al.*, 1999). Finally, reports support the notion that secretory vesicles destined to fuse with the apical cell surface transport both caseins and whey proteins (Devinoy *et al.*, 1995; Neville *et al.*, 1998; Ollivier-Bousquet, unpublished observation).

A large body of evidence supports the concept that local modifications of the lipid composition of membranous sub-domains by lipid-modifying enzymes also contribute to vesicular traffic. In line with this, phospholipase D and calcium-independent phospholipase A2 were reported to be involved in both the transport of milk proteins

from the ER to the Golgi and in the formation of secretory vesicles from the TGN (Boisgard and Chanat, 2000; Péchoux *et al.*, 2005), as was observed in other cell systems (Riebeling *et al.*, 2009; Schmidt *et al.*, 2010).

Trafficking steps within the secretory pathway of mammary epithelial cells and exocytosis of casein-containing vesicles with the plasma membrane might involve SNARE (soluble *N*-ethylmaleimide-sensitive fusion (NSF) attachment protein (SNAP) receptor) proteins, as already described in other cell types (Sollner *et al.*, 1993; Jahn and Scheller, 2006). To date, however, only a few studies have directly addressed the functions of SNARE proteins in mammary epithelial cells. One of these suggests that VAMP-8 (vesicle-associated membrane protein 8) may be involved in casein secretion (Wang *et al.*, 2007). On the other hand, SNAP-23 (synaptosomal-associated protein 23), syntaxin-3 and syntaxin-5, and Ykt6 have been described as being associated with lipid droplets (Boström *et al.*, 2007; Reinhardt and Lippolis, 2008). SNAP-23 is believed to play a role in homotypic fusion of intracellular lipid droplets. Moreover, the large amount of membrane necessary for MFG secretion by budding of the APM could be partly provided by exocytosis of casein-containing vesicles (Mather and Keenan, 1998; see Fig. 14.4). Recently, the endogenous expression levels of a large set of SNAREs were investigated in mouse mammary gland (Chat *et al.*, 2011). This study points to SNAP-23 as a potential central player for the coupling of casein and MFG secretion during lactation.

14.4.4 Hormonal Regulation of Milk Protein Secretion

Proteins destined for the cell exterior are secreted by either the constitutive or the regulated secretory pathway (Glombik and Gerdes, 2000; Morvan and Tooze, 2008). The fact that there is no substantial storage of newly synthesized milk proteins in mammary epithelial cells does not support the later hypothesis (Devinoy *et al.* 1995; Pauloin *et al.* 1997). On the other hand, hormones

including oxytocin and prolactin are able, at least *in vitro*, to increase casein secretion (secretagogue effect) in mammary epithelial cells from rabbits and rodents (see Ollivier-Bousquet, 1993, 1997). Prolactin seems to act on a late step of casein trafficking, possibly exocytosis. In contrast, oxytocin was reported to also accelerate the transport of newly synthesized proteins from the ER to the Golgi apparatus and to secretory vesicles (Lollivier *et al.*, 2006).

14.5 Amino Acid Transport by the Mammary Gland

Amino acids, extracted from interstitial fluid, are the major source of amino nitrogen for milk protein synthesis; therefore, a knowledge of mammary tissue amino acid transport mechanisms and their regulation is important if we are to understand fully the process of milk protein secretion. Such knowledge will help those wishing to manipulate milk protein content *via* dietary means. The uptake of amino acids across the basolateral membranes of mammary secretory cells, the major point of entry, is accomplished by an array of distinct transport mechanisms. The amino acid transporters differ from one another with respect to kinetics, substrate specificity and ion dependency; however, it is evident that they operate in a coordinated fashion to supply amino nitrogen to support milk protein synthesis.

The study of mammary epithelial amino acid transport is hampered by the relatively complex anatomy of the mammary gland. Nevertheless, the use of mammary tissue explants and the perfused mammary gland has enabled the transport of amino acids (using radiotracers) by mammary tissue to be characterized. Mammary explants are easy to prepare and have the advantage that the cellular architecture remains intact. Furthermore, the preparation of mammary explants does not require digestive agents: enzymes such as collagenase could ultimately alter the properties of membrane transport proteins. Although mammary tissue explants isolated from lactating animals are comprised of more than one cell type, it can be assumed that

the vast majority of the surface area of explants is that of the basolateral membrane of the secretory cells. Therefore, it is reasonable to assume that mammary tissue explants can be used to give a measure of amino acid transport across the blood-facing side of the mammary epithelium. The large tissue extracellular space associated with mammary explants does, however, place limitations on the design of experiments. The perfused mammary gland allows the transport of amino acids across the blood-facing aspect of the mammary epithelium to be measured under near physiological conditions: perfusates can be delivered to the gland with a flow and pressure profile similar to that found *in vivo*. The perfused mammary gland used in combination with a rapid, paired-tracer dilution technique allows the transport of amino acids to be measured over very short time courses (Mephram *et al.*, 1985; Calvert and Shennan 1996).

14.5.1 Mammary Tissue Amino Acid Transport Systems

The identification of individual amino acid transport systems is difficult on account of the fact that a single amino acid may be able to utilize several transport systems (see Barker and Ellory 1990). Furthermore, the difficulty in characterizing amino acid transport is compounded by the lack of specific inhibitors of amino acid transporters. In spite of these drawbacks, significant progress has been made towards identifying mammary tissue amino acid transport systems together with their putative molecular correlates. Mammary tissue amino acid transport mechanisms fall into two categories: Na⁺-dependent and Na⁺-independent systems.

14.5.1.1 Na⁺-Dependent Transport Mechanisms

Lactating mammary cells are able to concentrate free amino acids (particularly the non-essential ones) with respect to plasma, suggesting that there must be an input of free energy (Shennan *et al.*, 1997). It is apparent that several amino acid transport systems utilize the electrochemical Na⁺ gra-

dient to drive the movement of amino acids into mammary cells. The Na⁺-dependent mechanisms which have been identified in mammary tissue include systems A, ASC, X_{AG}⁻ and β.

System A prefers short-chain neutral amino acids as substrates and is characterized by its tolerance of *N*-methylated amino acids such as *N*-methylaminoisobutyrate (MeAIB). Indeed, Na⁺-dependent amino acid uptake inhibited by MeAIB is usually taken as a measure of transport *via* system A. It appears that mouse, rat and bovine mammary tissue possesses system A activity (Neville *et al.*, 1980; Baumrucker 1984; Verma and Kansal 1993; Shennan and McNeillie 1994a). Lopez *et al.* (2006) provided convincing evidence that SNAT2 may be the molecular correlate of system A in rat mammary tissue. In contrast, no evidence for the presence of system A at the blood-facing aspect of the guinea pig mammary gland could be found (Mephram *et al.*, 1985). The limited kinetic data available suggest that system A in lactating mammary tissue operates with relatively low affinity: the K_M of methionine and α-aminoisobutyric acid uptake *via* system A in mouse mammary tissue is 0.47 mM and 2.0 mM, respectively (Neville *et al.*, 1980; Verma and Kansal, 1993). Several lines of evidence suggest that system A is regulated by milk stasis, by starvation and by the stage of lactation (Neville *et al.*, 1980; Shennan and McNeillie 1994c; Verma and Kansal 1995). In accordance with these findings, SNAT2 mRNA expression, respectively, increases and decreases during lactation and weaning. Moreover, the abundance of SNAT2 mRNA is increased by oestrogen which may explain the high levels observed during pregnancy (Lopez *et al.*, 2006). System A activity is regulated by prolactin: treating animals with bromocriptine, a drug which inhibits prolactin secretion from the pituitary gland, markedly reduces the arteriovenous concentration differences of amino acids which are potential substrates of the A system (Vina *et al.*, 1981).

Mammary tissue from several species (e.g., bovine, mouse, guinea pig) has been shown to possess system ASC (Baumrucker 1985; Mephram *et al.*, 1985; Verma and Kansal 1993). It has been established in other tissues that this mechanism

cotransports neutral amino acids such as alanine, threonine and cysteine with Na^+ . System ASC in mammary tissue, like system A, operates with relatively low affinity: the K_M of methionine uptake by lactating mouse mammary tissue via system ASC is 0.46 mM (Verma and Kansal, 1993). At present, there appears to be a paucity of information on the regulation of mammary tissue amino acid transport via system ASC except for the finding that starvation gives rise to a large increase in system ASC activity in mouse mammary tissue (Verma and Kansal, 1995). There are at least two molecular isoforms of system ASC, both of which have been detected in mammary tissue. Thus, the expression of ASCT1 and ASCT2 mRNA has respectively been described in lactating rat and porcine mammary tissue (Aleman *et al.*, 2009; Laspiur *et al.*, 2009). The expression of both transcripts increases with the onset of lactation.

The transport of anionic amino acids by lactating mammary tissue has been extensively studied (Millar *et al.*, 1996, 1997a, b). The predominant, if not the only, pathway for L-glutamate and L-aspartate transport is a high-affinity ($K_M = 18 \mu\text{M}$ for L-glutamate), Na^+ -dependent system analogous to system X_{AG}^- (Kanai *et al.*, 1994). The Na^+ -dependent anionic amino acid carrier is very selective for anionic amino acids: it does not interact with neutral or cationic amino acids (Millar *et al.*, 1996, 1997b). An unusual feature of system X_{AG}^- is the ability to discriminate between the optical isomers of glutamate but not those of aspartate. The high-affinity anionic amino acid carrier is able to act as an exchange system as well as a cotransport mechanism suggesting that the transport of L-glutamate will affect the intracellular concentration of L-aspartate (and vice versa) (Millar *et al.*, 1997b). Several high-affinity anionic amino acid carriers, which have identity with system X-AG, have been cloned and characterized (e.g., see Kanai and Hediger, 1992; Pines *et al.*, 1992; Storck *et al.*, 1992). Three clones, EAAC1, GLAST and GLT-1, have been identified in rat mammary tissue (Martinez-Lopez *et al.*, 1999; Aleman *et al.*, 2009). However, the exact contribution of these isoforms to anionic amino acid transport across the basolateral membranes of mammary secretory cells is unknown.

Taurine, a nonprotein amino acid, is taken up by lactating rat and porcine mammary tissue *via* a high-affinity, Na^+ -dependent transport system analogous to system β (Shennan and McNeillie, 1994b; Bryson *et al.*, 2001). The mammary (Na^+ +taurine) cotransport system also requires Cl^- for maximal activity. System β has narrow substrate specificity: only β -amino acids such as taurine and β -alanine interact with the transporter. The activity of the rat mammary (Na^+ - Cl^- -taurine) cotransporter decreases as lactation progresses (Millar and Shennan 1999). In accordance with this, Aleman *et al.* (2009) have reported that the expression of rB16 (a cloned taurine transporter) mRNA decreases between early and peak lactation. The lactating gerbil mammary gland expresses a high-affinity (Na^+ -taurine) cotransport system which, unlike the rat mammary taurine transporter, is not dependent upon Cl^- (Shennan, 1995).

14.5.1.2 Na^+ -Independent Transport Mechanisms

System L has been identified in mouse, rat, guinea pig and bovine mammary tissue (Neville *et al.*, 1980; Mephram *et al.*, 1985; Verma and Kansal, 1993; Shennan and McNeillie, 1994c). System L is a Na^+ -independent transport mechanism that has wide substrate specificity. Indeed, system L may be the most important transport system for the uptake of essential neutral amino acids by the lactating mammary gland. Na^+ -independent amino acid transport sensitive to 2-aminobicycloheptane-2-carboxylic acid (BCH) is taken as a measure of system L activity. It is generally accepted that system L can act as an amino acid exchange mechanism. Accordingly, methionine uptake by mouse mammary gland *via* system L can be *trans*-accelerated by intracellular amino acids. However, amino acid efflux from rat mammary tissue, *via* system L, is not *trans*-stimulated by extracellular amino acids, suggesting that the transporter operates with asymmetric kinetics which could favour the retention of substrates within the gland. System L has been localized to the basolateral aspect of the lactating rat mammary gland (Shennan *et al.*, 2002). There are at least four molecular isoforms of system L

(LAT 1–4), two of which have been described in the mammary gland. Thus, LAT1 and LAT2 mRNA are expressed in the rat mammary gland (Shennan *et al.*, 2002; Aleman *et al.*, 2009). Interestingly, Aleman *et al.* (2009) have shown that LAT1 mRNA expression in rat mammary tissue markedly increases during lactation. LAT mRNA is also expressed in the mouse and bovine mammary gland (Rudolph *et al.*, 2007; Finucane *et al.*, 2008; Connor *et al.*, 2008). The expression of LAT1 mRNA increases during the transition from pregnancy to lactation and is also up-regulated by milking frequency.

The transport of cationic amino acids by lactating mammary tissue is a Na^+ -independent process (Baumrucker 1984; Shennan *et al.*, 1994b; Hurley *et al.*, 2000). The pathway for lysine and arginine uptake by bovine mammary tissue is not affected by replacing extracellular Na^+ and appears to be relatively specific for cationic amino acids. On this basis Baumrucker (1984) concluded that cationic amino acids are transported via system y^+ . There is evidence suggesting that CAT-1 may be responsible for system y^+ activity in rat mammary tissue (Aleman *et al.*, 2009). CAT-1 mRNA expression is low in mammary tissue isolated from pregnant rats and increases with the onset of lactation (Aleman *et al.*, 2009). Transcripts for two cloned Na^+ -independent cationic amino acid transporters, CAT-1 and CAT-2B, have been detected in lactating porcine tissue (Laspiur *et al.*, 2009). However, it appears that the expression of both transcripts is relatively unaffected by the stage of lactation. Cationic amino acid uptake by lactating rat mammary tissue is also facilitated by a transporter which interacts with neutral amino acids such as glutamine and leucine (Shennan *et al.*, 1994a; Calvert and Shennan 1996). Thus, certain neutral amino acids respectively inhibit and stimulate lysine uptake by and efflux from rat mammary tissue. It is likely that this pathway is y^+L : y^+L -LAT1 mRNA has been detected in lactating rat mammary tissue (Boyd, Kudo and Shennan, unpublished).

System T, a mechanism specific for aromatic amino acids, has been described in lactating mouse mammary tissue (Kansal and Kansal,

1996). Thus, the moiety of tyrosine uptake by mouse mammary tissue which is not dependent upon extracellular Na^+ and is not sensitive to BCH has been ascribed to system T. This mechanism operates with low affinity; the K_M for tyrosine transport is approximately 15 mM.

14.5.1.3 Volume-Activated Amino Acid Transport

To survive, cells have to regulate their volume within relatively narrow limits (see Hoffmann and Simonsen, 1989, for a review). Cell membranes are very permeable to water which means that cell volume, otherwise termed the cellular hydration state, will be determined by the osmolarity of the extracellular fluid and by the intracellular content of osmotically active solutes. The cellular hydration state can be changed as a consequence of anisotonic conditions, cellular accumulation of solutes or oxidative metabolism. Cells are able to regulate their volume following swelling or shrinking. Cell volume regulation involves the transmembrane movement of solutes together with osmotically obliged water (Hoffmann and Simonsen, 1989).

A knowledge of volume-regulatory processes in mammary tissue is of particular importance given that cell volume changes markedly affect the rate of milk protein synthesis (Millar *et al.*, 1997a). It has been established that cell swelling activates the transport of amino acids in mammary tissue (Shennan *et al.*, 1994; 1996). Thus, cell swelling, induced by a hypoosmotic shock, increases the efflux of amino acids such as taurine and glycine via a pathway which has the characteristics of a channel rather than a carrier. The swelling-induced amino acid efflux pathway appears to be situated in the blood-facing aspect of the mammary epithelium (Calvert and Shennan, 1998). The volume-activated efflux of taurine is dependent upon the extent of cell swelling and can be inhibited by a number of pharmacological agents such as niflumic acid (Shennan *et al.*, 1996). There is the strong possibility that volume-activated amino acid efflux may participate in mammary cell volume regulation given that amino acids are concentrated within mammary cells with respect to plasma.

14.5.2 Transport and Metabolism of Peptides

Although free amino acids are the major source of amino nitrogen for milk protein synthesis, it is evident that the uptake of several essential amino acids is less than their output in milk, suggesting that other circulating forms of amino acids, such as peptides, may be available for casein production (e.g., see Backwell *et al.*, 1996). In this connection, it has been demonstrated that the goat mammary gland is able to use intravenously administered peptides for milk protein synthesis (Backwell *et al.*, 1994, 1996). The *in vivo* studies were unable to show whether the mammary gland transported peptides intact or whether the peptides were hydrolysed extracellularly followed by uptake of the liberated amino acids. It has been shown, albeit indirectly, that the mouse mammary tissue does not significantly hydrolyse peptides extracellularly but is able to transport peptides intact (Wang *et al.*, 1996). On the other hand, studies using the rat mammary gland as a model suggest that mammary tissue is able to both transport and hydrolyse dipeptides (Shennan *et al.*, 1998, 1999). It is evident that the perfused lactating rat mammary is able to transport dipeptides which are resistant to hydrolysis (i.e., D-Phe-L-Gln; D-Phe-L-Glu); the nature of the pathway remains to be identified precisely. However, it is apparent that the capacity of the rat mammary gland to transport peptides intact across the basolateral pole of the epithelium is relatively limited (Shennan *et al.*, 1998). Transcripts for two proton-dependent peptide transporters, namely, PepT1 and PepT2, have been identified in lactating rat and human mammary epithelial cells (Alcorn *et al.*, 2002; Groneberg *et al.*, 2002; Gilchrist and Alcorn, 2010). PepT2 protein is localized in the apical membrane of rat ductal epithelial cells and is therefore not in a position to facilitate the uptake of peptides from the circulation. Rather, it is likely that PepT2 provides a route for the reuptake of peptides from milk as postulated by Shennan and Peaker (2000).

Rat mammary tissue is able to extensively hydrolyse peptides extracellularly: this has been

demonstrated using both direct and indirect experimental approaches (Shennan *et al.*, 1998; 1999). Anionic dipeptides presented to rat mammary tissue can *trans*-stimulate D-aspartate efflux *via* the high-affinity anionic amino acid carrier. If it is accepted that dipeptides do not interact directly with the amino acid carrier, then it can be assumed that anionic amino acids, produced as a consequence of extracellular hydrolysis, act to stimulate D-aspartate efflux. In this connection, it has been shown that mammary tissue is capable of hydrolysing a variety of aminoacyl-*p*-nitroanilides (peptide analogues). Quantitatively, it appears, at least in the rat, that hydrolysis of peptides followed by uptake of the free amino acids may be more important than the transport of peptides (Shennan *et al.*, 1998).

The identity of the peptidases involved in the extracellular hydrolysis of peptides by mammary tissue has not been established. However, γ -glutamyltranspeptidase appears to be involved given that glutathione can stimulate D-aspartate efflux from rat mammary tissue in a fashion sensitive to acivicin (Shennan *et al.*, 1998). The hydrolysis of glutathione may be an important route for providing the mammary gland with cysteine. There is evidence to suggest that aminopeptidase N plays a role in providing free amino acids for protein synthesis in the goat mammary gland (Liu *et al.*, 2010).

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