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SYNTHESIS AND PROCESSING OF MILK PROTEINS

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Until recently the major interest in milk proteins derived from the observation that the limited number of major protein constituents present were organ specific, and that their expression was modulated by a well defined combination of both peptide and steroid hormones (for reviews see Topper & Oka (1); Banejee (2); Rosen (3)), thus providing a potentially exciting system with which to study the manner in which hormones modulate gene expression.

Experiments concerning the intracellular mechanisms involved in the synthesis and processing of secretory proteins are based fundamentally on the pioneering work of Siekevitz & Palade (4) who proposed that membrane-bound polyribosomes represented the site of synthesis of secretory proteins, whereas proteins required for intra-cellular functions (the house-keeping proteins) were synthesized on free polyribosomes. Studies designed to substantiate this concept have too often evolved around

identification of the site of synthesis of immunoglobulin producing tumour-derived cell lines, which on careful comparison have produced conflicting data, both for (Ref. 5,6) and against (Ref. 7,8) this concept. However, studies using normal tissue particularly liver have gone some way to substantiate these concepts, thus there has accumulated a considerable volume of evidence which demonstrates that the major secretory component of liver, serum albumin, is synthesized primarily on membrane-bound polyribosomes, whereas ferritin, the major intracellular iron-storage protein, is synthesized primarily on free polyribosomes (see Ref. 9,10,11,12).

It has long been accepted that milk proteins are synthesized primarily on membrane-bound polyribosomes (for reviews see Denamur (13); Craig & Campbell (14)). However, in spite of the use of tissue from the normal lactating mammary gland, results concerning the precise proportion of total milk protein mRNA species associated with free or membrane-bound polyribosomes have been conflicting. Moreover as with other secretory protein systems, experiments have generally been designed to determine only the relative intracellular distribution of secretory protein or 'abundant' mRNA species and have ignored the relative distribution of the less abundant and scarce mRNA species which direct the synthesis of the many proteins not secreted by the cell. Our own preliminary observations using lactating guinea-pig mammary gland tissue, showed that after subcellular fractionation 85% of the recovered polyribosomal RNA was associated with the membrane-bound fraction, and the remainder with the free polyribosomal fraction (Ref. 15). Analysis of the ability of poly(A)-containing RNA associated with both polyribosome fractions to direct casein synthesis in a Krebs II ascites cell-free protein synthesizing system as judged by antibody precipitation, although confirming that the membrane-bound polyribosomes, were undoubtedly the predominant site of guinea-pig milk protein synthesis, also revealed considerable casein mRNA activity associated with the free polyribosome fraction. These observations were in some contrast to those of Houdebine and Gaye (16) working with the lactating ewe mammary gland, who reported the almost exclusive synthesis of casein on membrane-bound polyribosomes, as judged by the translation of casein mRNA in a reticulocyte lysate cell-free protein synthesizing system.

Since these early observations, techniques used to isolate polyribosomal fractions have improved (17), whilst the introduction of molecular hybridisation techniques, using specific cDNA probes for the quantification of specific mRNA species, has eliminated artefacts due primarily to the preferential translation of certain mRNA

species, a function of the ionic conditions used for cell-free protein synthesis (Ref. 18,19). Consequently, a recent re-evaluation by Houdebine (20) of the distribution of casein mRNA between free and membrane-bound polyribosomes during lactogenesis in the rabbit, showed that 95% and 5% of total polysomal casein mRNA was found in membrane-bound and free polyribosomes respectively, whilst a considerably higher percentage of casein mRNA was associated with the free polyribosomes fraction in the pseudopregnant rabbit in the absence of prolactin.

Our own studies were designed not just to re-evaluate our previous observations, but to analyse the general distribution of polyribosome-associated mRNA species, using a combination of mRNA directed cell-free protein synthesis, polyribosome 'run-off' in the presence of an inhibitor of initiation, and comparative complexity analyses of the poly(A)-containing RNA species isolated from the free polyribosomes, membrane-bound polyribosomes and the post-nuclear supernatant of the lactating guinea-pig mammary gland. This approach (Ref. 19) demonstrated unequivocally that although as much as 40% of the poly(A)-containing RNA species associated with the free polyribosomes comprised of the abundant or milk protein mRNA species, the remainder were divided into two well defined groups, one comprising of a relatively abundant group of poly(A)-containing RNA species (450-550), and the other a large group (8000-12000) poly(A)-containing RNA species each present in only a few copies. A similar analysis of the total cytoplasmic poly(A)-containing RNA population, confirmed this general distribution, though the relative proportion of each group differed slightly, due primarily to the fact that 55% of the total population comprised of the abundant mRNA population representing the milk proteins. However, analysis of the membrane-bound poly(A)-containing RNA population revealed a considerably less complex profile. As expected the abundant milk protein mRNA species were predominant, representing over 70% of the total population, whereas the remaining poly(A)-containing RNA species appeared to comprise of a single population (2500-3500 species) of poly(A)-containing RNA molecules, a markedly less complex population than that associated with the free polyribosomes. These results supported by polyribosome 'run-off' experiments using the wheat germ cell-free protein synthesizing system *in vitro* (Ref. 19) clearly demonstrate that in the lactating guinea-pig mammary gland, there exists a very sharp functional distinction between the two polyribosomal classes. Furthermore, 'run-off' experiments conducted in the presence of aurin tricarboxylic acid (an inhibitor of initiation), suggest that significant levels of milk protein mRNA associated with the free polyribosomal preparation, may

well be present as mRNP particles, and therefore causing an over estimation of the true level of milk protein mRNA species associated with the free polyribosomal preparation. However assuming that at any one time 80-85% of the total polyribosome population is associated with the endoplasmic reticulum, then in excess of 90% of the abundant milk protein mRNA population is in the form of membrane-bound polyribosomes. What do the remainder represent? It is now well established, that in addition to secretory proteins, certain integral membrane glycoproteins are also synthesized on membrane-bound polyribosomes (Ref. 22,23,24,25), thus it seems probably that the remainder of the poly(A)-containing RNA species associated with membrane-bound polyribosomes, may well direct the synthesis of either structural membrane proteins or alternatively some of the many enzymes (e.g. casein kinases) required during subsequent post-translational events involved in the secretory pathway.

Although it has been established that in the lactating mammary gland there exists a very clear functional distribution between the two polyribosome classes (Ref. 19, 20), it is of considerable importance to consider how these two types of polyribosome arise, and how those involved in secretory protein synthesis become attached to membranes, resulting ultimately in the synthesized proteins passing through the membranes into the cisternae of the endoplasmic reticulum from whence they are transported to the Golgi apparatus, packaged into secretory granules and finally released by fusion with the plasma membrane - for review see Palade (21).

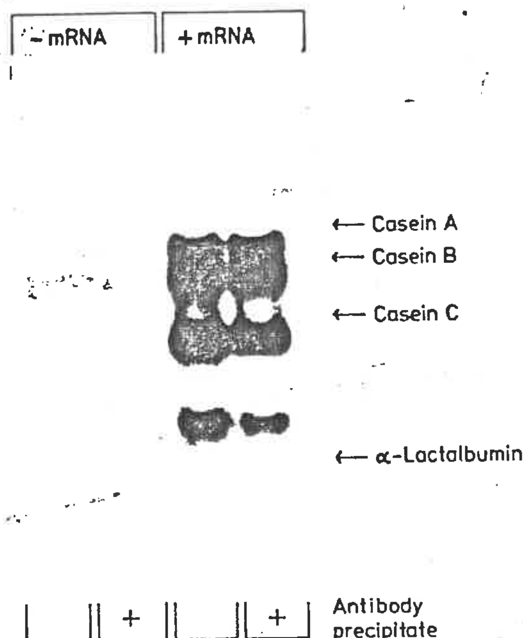
As there seems little evidence to suggest that free or membrane-bound polyribosomes contain different pools of 40S and 60S ribosomal subunits (Ref. 26), it is generally accepted that some form of receptor site may exist on the endoplasmic reticulum. This will then be recognised either by the mRNA itself, or by protein(s) associated with the mRNA, or alternatively the nascent peptide. Evidence for the direct interaction of the mRNA with the membrane is conflicting (Ref. 27,28,29) though experiments performed _in vivo_ suggest that the mRNA does not contribute significantly to the maintenance of interaction between bound polyribosomes and the membrane of the endoplasmic reticulum (Ref. 30,31). Recently, evidence has been presented (Ref. 32) which suggests that two proteins, both integral components of the rough endoplasmic reticulum appear to be related to bound polyribosomes, and therefore may be considered as possible candidates for the putative polyribosome membrane receptor site. However, evidence that the specificity of attachment, though not necessarily the mechanism of attachment resides in the nascent polypeptide is more

compelling. This concept originated from observations by Milstein (33), who showed that when mRNA directing the synthesis of immunoglobin light-chain was translated in a cell-free protein synthesizing system that contained fragments of endoplasmic reticulum, and one that did not, then the product of the latter was larger, owing to the presence of an N-terminal peptide extension. These observations led to the formulation of the 'Signal' hypothesis (Ref. 33,34), which postulates the existence of a metabolically short-lived N-terminal extension to cell proteins to be segregated in membrane-bound compartments. Thus the presence of a 'Signal' pre-peptide would give rise to polyribosomes capable of attachment to the endoplasmic reticulum, via an interaction of the signal peptide with membrane receptor sites. Its absence would require complete translation of the mRNA on free polyribosomes. This hypothesis has now been elaborated (Ref. 6,35) and an accumulation of evidence has demonstrated that so far, with the major exception of the egg white protein, ovalbumin (Ref. 36), secretory proteins are synthesized with a hydrophobic signal sequence, of 18-30 amino-acid residues in length (Ref. 37,38,39,40,41, 42,43). Moreover, ingenious experiments ultilizing mRNA directing the synthesis of secretory or membrane proteins, in conjunction with cell-free protein synthesizing systems (synchronised using 7-methylguanosine 5'-phosphate) in the presence or absence of membrane fragments, have provided evidence that *in vitro*, membrane-bound ribosomes engaged in the synthesis of secretory proteins, are derived from free polyribosomes (Ref. 25,44).

It seems reasonable to predict that milk proteins should provide a suitable system with which to study intricate intracellular secretory mechanisms, particularly as mRNA species directing their synthesis have proved both easy to isolate in large quantities, and also highly active in a variety of cell-free protein synthesizing systems (see Craig & Campbell (14), Rosen (3)). Unfortunately this has not been the case. Although we demonstrated some time ago (Ref. 45), that the guinea-pig whey protein α-lactalbumin was synthesized in cell-free protein synthesizing systems lacking endoplasmic reticulum as pre-α-lactalbumin, similar positive evidence for the synthesis of pre-caseins has until recently been conspicuously lacking.

Figure 1, demonstrates the mRNA dependent synthesis of the four major guinea-pig milk proteins in the wheat germ cell-free system, in the presence of [^{35}S]methionine. The products have been identified using antibody precipitation, separated by SDS polyacrylamide gel electrophoresis (Ref. 46), and then visualised using fluorography (Ref. 47). The arrows denote the relative

<u>In vitro</u> synthesis of Guinea Pig milk proteins in the Wheat Germ cell-free system.



position on the gel of the marker proteins isolated from guinea-pig milk, whilst the dark bands denote the position of the <u>in vitro</u> synthesized antibody precipitable products. As can be clearly seen, essentially all the synthesized proteins are antibody precipitable, and none co-electrophorise with the marker proteins. One <u>in vitro</u> product (smallest) appears larger than the equivalent marker protein (α-lactalbumin), this in fact is pre-α-lactalbumin (Ref. 45), whilst the remainder all appear smaller than the equivalent marker caseins. Such observations appear to be common to all published data concerning the <u>in vitro</u> synthesis of caseins, as independent of the source of mRNA, the <u>in vitro</u> products all appeared to be either the same size or smaller than the authentic peptides (Ref. 45,48,49,50). However, the application of microsequencing techniques (Ref. 39) to the radiolabelled <u>in vitro</u> synthesized caseins has now shown that in spite of their anomalous mobility when analysed by SDS-polyacrylamide gel electrophoresis, all ovine (Ref. 51) and rat caseins (Ref. 3) examined so far, contain the 'Signal' NH_2-terminal peptide typical of secretory proteins.

Evidence for the presence of precursors for guinea-pig caseins, is indirect, and has been derived from compara-

tive analysis of mRNA directed milk protein synthesis in protein synthesizing systems containing either intact endoplasmic reticulum (the oocyte) or fragments thereof. Thus analysis of milk proteins synthesized in the Krebs II ascites cell-free system which contains membrane fragments, reveals that some of the pre-α-lactalbumin has been processed into the mature protein (Ref. 45,52) whilst additional products are present representing processing of the caseins. Examination of the oocyte products reveals the presence of only the processed proteins, whereas examination of the wheat germ or reticulocyte products shows only the unprocessed proteins. Evidence that this is both a membrane function and a NH_2-terminal cleavage, has been obtained by comparison of the labelling pattern of the milk protein mRNA directed products, synthesized in the wheat germ cell-free system in the presence and absence of dog pancreas endoplasmic reticulum using either [^{35}S]Met-$tRNA_f$, [^{35}S]Met-tRNA or [^{35}S]-Methionine as the radiolabelled precursors (Ref. 52). In the presence of the latter, and added membranes, there is a marked change in the mobility of the caseins. A similar change in mobility is observed with [^{35}S]Met-tRNA in the presence of membranes. However, in the presence of [^{35}S]Met-$tRNA_f$ which will only label the initiating methionine, the addition of membrane essentially eliminates all bands from the gel, strongly suggesting that guinea-pig caseins are not only processed at the level of the endoplasmic reticulum, but that this is the result of an NH_2-terminal peptide cleavage. Moreover regardless of the cell-free protein synthesizing system, or the source of the added endoplasmic reticulum, whether it be from the guinea-pig mammary gland or from the dog pancreas, we have yet to obtain in any in vitro system caseins with the same mobility on SDS-polyacrylamide gels as the final secreted products in vivo. All guinea-pig caseins are phosphorylated (Ref. 56), a post-translational event known to occur in the Golgi apparatus (Ref. 53). As it seems probable that the presence of high levels of phosphate will affect the mobility of proteins on SDS polyacrylamide gels, it seems reasonable to conclude that the anomalous mobility of the in vitro synthesized caseins may well be due to the absence of phosphorylation in vitro. This conclusion is supported by our inability so far to phosphorylate (using [γ-^{32}P]ATP) our in vitro synthesized caseins, independent of the cell-free system of choice though the wheat germ in particular shows high levels of endogenous protein kinase activity.

Although we have demonstrated that milk protein processing is a membrane function, it is important to determine the intracellular fate of the products, and also to determine whether or not the passage of proteins

into the cisternae of the endoplasmic reticulum is directly linked to the translational event. Experiments designed to determine the intracellular fate of secretory and non-secretory proteins synthesized by the Xenopus oocyte (Ref. 54) demonstrated that both milk protein and albumin mRNA species directed the synthesis of vesicularised products, as judged by the resistance of the newly synthesized proteins to proteinase digestion in the absence of detergent. A further series of experiments involving the microinjection of [^{35}S]methionine labelled milk protein precursor proteins synthesized by the wheat germ cell-free system into the oocyte, showed that less than 2% of these entered vesicles. Moreover, unlike the vesicularised proteins which are extremely stable, the unprocessed milk proteins were rapidly degraded by oocyte peptidases. In sharp contrast, the microinjection of ovalbumin synthesized in the wheat germ (this has no signal peptide) into the oocyte, resulted in minimal degradation of the protein (Ref. 55).

These observations are interesting in that (i) they demonstrate in a essentially _in vivo_ situation, the absolute requirement for active ongoing translation before selective compartmentalisation of secretory proteins may occur, even in the presence of the signal peptide and (ii) raise the possibility for an alternative or additional role for the signal peptide, in that should secretory proteins bearing signal sequences arise in the wrong compartment of the cell, then these are rapidly degraded, effectively preventing the accumulation of the primary translation products.

The results of our investigations and those of others into the intracellular mechanisms involved in milk-protein secretion, confirm that a very clear functional distinction exists between the two classes of polyribosomes. Precisely how these arise has still to be established. Our evidence is consistent with the initiation of all protein synthesis on free polyribosomes, followed by the attachment of a discrete population of polyribosomes to the endoplasmic reticulum. It seems reasonable to predict that the nascent 'Signal' sequence plays a role in the specificity of these events, involving (i) the initial interaction between the 'free' polyribosome and the putative membrane receptor sites, and (ii) possibly at a different level preventing the miscompartmentalisation of those secretory proteins bearing such a sequence.

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