Differential expression of α -lactalbumin and casein genes during the onset of lactation in the guinea-pig mammary gland

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1. The expression of α -lactalbumin and casein genes was examined in guinea-pig mammary tissue taken from animals both pre- and post-partum. 2. Analysis of total RNA by RNA excess hybridization with sequence-specific complementary DNA probes demonstrated that α -lactalbumin mRNA was present late in pregnancy, and that maximum concentrations were present at parturition. Casein gene transcripts were absent late in pregnancy (62 days), but by parturition were present at concentrations identical to those found at all time points examined throughout lactation. 3. Studies using mammary explants in organ culture showed that tissue from pregnant animals, or animals at parturition, synthesized and secreted only α -lactalbumin. After parturition, at the onset of casein synthesis, differential rates of secretion of α -lactalbumin and the caseins were observed. 4. The results are discussed in terms of the multiple intracellular mechanisms involved in the regulation of milk protein gene expression in the guinea-pig mammary gland.

The growth and differentiation of the mammary gland is regulated by an intricate combination of steroid and peptide hormones (see Wood *et al.*, 1975; Banerjee, 1976). In particular it is now established that the synthesis of milk proteins, the major protein products of the fully differentiated lactating mammary gland, is stimulated by prolactin, a peptide hormone, and glucocorticoids (see Topper & Oka, 1974; Devinoy *et al.*, 1978; Matusik & Rosen, 1978; Ganguly *et al.*, 1979). Progesterone appears to regulate milk protein gene expression in an inhibitory manner (Assairi *et al.*, 1974; Houdebine & Gaye, 1975; Rosen *et al.*, 1978; Matusik & Rosen, 1978).

Studies on mammary tissue from the rabbit (Shuster *et al.*, 1976) and rat (Rosen *et al.*, 1975, 1978; Nardacci *et al.*, 1978; Nakhasi & Qasba, 1979) during different stages of gestation through to parturition demonstrate the presence of significant levels of milk protein mRNA sequences by mid-

pregnancy. Unlike the rabbit, mouse and rat, where considerable mammary development has occurred by mid-pregnancy (see Anderson, 1974), the most rapid period of proliferative growth in guinea-pig mammary glands occurs immediately after parturition (Nelson *et al.*, 1962). Consequently it was of interest to determine whether milk protein gene expression in the guinea-pig could be detected during pregnancy, as reported in other animal systems, or if, consistent with the mammary developmental pattern, the genes were only expressed immediately before or after parturition.

Guinea-pig milk contains four major organ specific proteins, the whey protein α -lactalbumin (Brew, 1972) and three caseins (Craig *et al.*, 1976, 1978). We have previously described the isolation of a poly(A)-containing RNA population from the lactating mammary gland which directs the synthesis of the primary milk protein polypeptides *in vitro* (Craig *et al.*, 1976, 1979a,b).

In this paper we show that, in the guinea-pig, α -lactalbumin is synthesized late in pregnancy, and that the maximum concentration of α -lactalbumin RNA transcripts is present at parturition. In contrast, casein RNA transcripts are not present in detectable amounts in the late-pregnant gland, but at parturition are present at a concentration similar to that found in the fully lactating gland. However,

Abbreviations used: cDNA, complementary DNA; SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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casein synthesis is not apparent until after parturition. The results are discussed in terms of the multiple intracellular mechanisms required to regulate milk protein gene expression.

Materials and methods

Preparation of solutions and glassware

Preparation of buffers, scintillation fluids, all recrystallizations and distillation procedures were as described by Craig *et al.* (1976, 1979a).

Glassware used for cDNA preparations and nucleic acid hybridizations was acid washed in 1 M-HCl, silicone-treated, washed with double-distilled water and then sterilized overnight at 180°C before use.

Materials

All tissue culture medium was obtained from Gibco Biocult; benzyl penicillin and streptomycin sulphate were from Glaxo; bovine insulin, phenyl-methylsulphonyl fluoride and poly(U) were from Sigma. Hydrocortisone acetate was from Rousell Laboratories. Plastic tissue culture dishes (Falcon) were from Becton and Dickinson.

All chemicals, isotopes, animals, solvents and enzymes were from sources previously described (Craig *et al.*, 1976, 1979a,b, 1981; Bathurst *et al.*, 1980a). Avian-myeloblastosis-virus reverse transcriptase (lot no. G-379) was provided by Dr. J. W. Beard, Life Sciences Inc., St. Petersburg, FL 33707, U.S.A. Dunkin Hartley guinea-pigs (post-partum) were obtained from A. Tuck and Son, Battlesbridge, Essex, U.K. Timed pregnancy animals (see Nelson *et al.*, 1962) were bred in our own animal house or were the gift of Dr. A. Firth, St. George's Hospital Medical School, Tooting, London SW17 0RE, U.K.

Isolation of total cellular RNA and total poly(A)-containing RNA

Total RNA was extracted from frozen guinea-pig mammary tissue as described by Hall *et al.* (1979). Residual RNA was then removed with ribonuclease-free deoxyribonuclease as described previously (Bathurst *et al.*, 1980a).

Preparation of ³H-labelled DNA complementary to milk protein mRNA sequences

High-specific-radioactivity ³H-labelled DNA complementary to total post-nuclear poly(A)-containing RNA from the lactating mammary gland of the guinea-pig was synthesized as described previously (Craig *et al.*, 1979a) by using [³H]dCTP (22 Ci/mmol) as the radiolabelled deoxyribonucleoside triphosphate. An abundant DNA popula-

tion complementary to all the milk protein mRNA sequences was isolated from the total cDNA population by limited hybridization to the homologous RNA preparation followed by S₁ nuclease digestion (see Craig *et al.*, 1979a).

³H-labelled cDNA complementary to individual milk protein mRNA sequences was isolated from the total post-nuclear cDNA population by hybridization to milk protein cDNA cloned into the plasmid pAT 153 (see Craig *et al.*, 1981), then immobilized on paper in a similar manner to that described by Alwine *et al.* (1977). Briefly, the following procedure was employed for recombinant plasmids containing cDNA sequences for caseins A, B and C, and α -lactalbumin. Partially *Hae*III-restricted plasmid DNA (20 μ g) was immobilized on 1 cm² filters of Whatman 540 paper as described by Wahl *et al.* (1979). The filter-bound DNA specific for each milk protein (i.e. four filters) were placed in a plastic scintillation vial insert, and immersed in 100 μ l of 20 mM-Hepes (pH 7.6) containing 0.6 M-NaCl, 1 mM-EDTA, 0.1% (w/v) SDS, 20 μ g of poly(A)/ml, 60% (v/v) formamide, and 10⁶ c.p.m. of the total post-nuclear ³H-labelled cDNA preparation. The inserts were capped, immersed in a water-bath, and incubated at 25°C overnight. The filters were then each placed into fresh inserts, and the cDNA sequences that had not hybridized were removed by four separate 5 min incubations at 25°C in 3 ml of 20 mM-NaCl/8 mM-sodium citrate/1 mM-EDTA/0.2% SDS in 60% (v/v) formamide. Hybridized cDNA sequences were eluted from the filters by three separate incubations in 100 μ l of 0.1 M-NaOH/1 mM-EDTA for 5 min at 37°C. The washings were then pooled, neutralized by the addition of 0.1 vol. of 1 M-sodium acetate, pH 4.5, and the [³H]cDNA was recovered by ethanol precipitation in the presence of 50 μ g of *Escherichia coli* tRNA. The precipitated nucleic acid was washed once in absolute ethanol, dried briefly *in vacuo*, dissolved in double-distilled water and stored at -70°C. Using these conditions 4.1% of the total [³H]cDNA was recovered with an α -lactalbumin cDNA plasmid (pgp K-9) and 5.5%, 8.3% and 4.1% respectively with caseins A, B and C cDNA plasmids (pgp N-33, pgp K-27, and pgp O-22; see Craig *et al.*, 1981).

RNA excess hybridization procedures

All hybridization procedures were performed at 70°C in 0.24 M-sodium phosphate buffer, pH 6.8, containing 0.2% SDS and 1 mM-EDTA. Hybridization details and determination of the percentage of cDNA hybridized by using S₁ nuclease were as described previously (Craig *et al.*, 1979a). In some instances the amount of cDNA was kept constant but the volume of the reaction was increased to 10 or 20 μ l to ensure that in no instance was the cDNA in excess over the milk protein mRNA.

Incorporation of [35 S]methionine into proteins synthesized by mammary gland explants in organ culture

Pregnant or lactating guinea-pigs were killed by cervical dislocation, and glands were dissected aseptically into ice-cold Eagles minimal essential medium (MEM) buffered with 10 mM-Hepes, pH 7.6. Explants (0.5–0.8 mg) were removed from the glands, and each was placed onto a 12.5 μ l droplet of methionine-free Eagles minimal medium containing 0.5 mCi of [35 S]methionine (800–1300 Ci/mmol)/ml on a flat plastic surface (5 cm vented tissue culture dish; 6–10 explants per dish). The dishes were then incubated at 37°C for 2 h in an atmosphere of air/CO₂ (19:1). The medium surrounding each explant was then carefully removed, diluted with 37.5 μ l of 10 mM-Tris/HCl, pH 7.4 at 20°C, containing 0.25 M-sucrose, 50 mM-NaF, 1 mM-phenyl-methylsulphonyl fluoride and 1% (v/v) Triton X-100

(explant buffer) and stored at –20°C. Each explant was then homogenized in 50 μ l of explant buffer, with a glass/glass hand homogenizer at 4°C. Insoluble debris was removed by centrifugation on a Beckman Microfuge for 2 min, and the supernatant was retained and stored at –20°C. Milk proteins in the tissue extract or medium were then identified by a combination of double antibody precipitation, electrophoresis on 12.5% polyacrylamide gels and fluorography, as described elsewhere (Craig *et al.*, 1979b; Pascall *et al.*, 1981).

Results

Total RNA was isolated from the glands of a series of animals, some pregnant, some in labour, and some at various times post-partum. The presence of milk protein RNA transcripts was initially determined by RNA excess hybridization using an

Fig. 1. Analysis of milk protein synthesis by explants of mammary tissue in culture, taken from guinea-pigs 50 and 60 days into pregnancy

[35 S]Methionine-labelled polypeptides synthesized and secreted by mammary explants were analysed by a combination of polyacrylamide-gel electrophoresis, and antibody precipitation using a mixture of antisera raised against guinea-pig α -lactalbumin and caseins A, B, and C (see the Materials and methods section). Samples were as follows: tracks 1–4, total 35 S-labelled polypeptides present in tissue (tracks 1 and 2) or secreted into medium (tracks 3 and 4). Tracks 5–8, antibody-precipitable 35 S-labelled polypeptides from tissue (tracks 5 and 6) or from medium (tracks 7 and 8). Tracks 1, 3, 5 and 7, tissue from 50 day pregnant animals; tracks 2, 4, 6 and 8, tissue from 60 day pregnant animals. Arrows denote the relative electrophoretic mobility of guinea-pig caseins A, B, C, and α -lactalbumin isolated from guinea-pig milk (see Craig *et al.*, 1976, 1978).

abundant ^3H -labelled cDNA probe representative of all the milk protein mRNA sequences (Craig *et al.*, 1979a). This showed that 2 days before parturition (62 days pregnant) only limited protection of the hybridization probe had occurred by a R_0t value of $100\text{ mol of nucleotide} \cdot \text{s}^{-1} \cdot \text{litre}^{-1}$, indicating the absence of most, if not all, milk protein RNA transcripts. In contrast, analysis of the RNA from animals at parturition, and 8 h, 24 h, and 5 days post-partum, showed that the relative concentration of milk protein sequences in all these tissues was very similar to the concentration present in the 7 day post-partum gland (results not shown). In no instance was the relative concentration less than half that of the fully lactating gland, which contains as many as 10^5 copies of each sequence per cell (see Bathurst *et al.*, 1980b).

In order to determine whether these sequences represented, particularly in the earlier time points, active milk protein mRNA, engaged in milk protein synthesis *in vivo*, we have examined the spectrum of [^{35}S]methionine-labelled peptides synthesized and secreted by tissue explants in organ culture, using a combination of antibody precipitation and SDS/polyacrylamide-gel electrophoresis. This approach demonstrated that tissue removed from animals 50 and 60 days pregnant synthesized a peptide of the expected mobility of α -lactalbumin (Fig. 1, tracks 1 and 2) and that this was also the predominant secreted protein in the medium (Fig. 1, tracks 3 and 4). Antibody precipitation of the total [^{35}S]methionine-labelled polypeptides using a mixture of antisera raised against guinea-pig α -lactalbumin and caseins confirmed that this was α -lactalbumin (Fig. 1, tracks 5–8). No peptide compatible with the electrophoretic mobility of the guinea-pig caseins was apparent either in the tissues or medium. Examination of the [^{35}S]methionine-labelled peptides synthesized and secreted by mammary explants taken from animals at parturition and 10 h and 72 h post-partum showed (Fig. 2) that, of the milk proteins, only α -lactalbumin could be detected in the tissue and secretions of the animal at parturition (Fig. 2, tracks 1 and 4). By 10 h post-partum, only α -lactalbumin was apparent in the tissue, but all the guinea-pig caseins and α -lactalbumin were present in the medium, indicating rapid secretion (Fig. 2, tracks 2 and 5). Examination of the peptides synthesized by the fully lactating gland (72 h post-partum) showed that all the milk proteins could be identified in the tissue and secretions (Fig. 2, tracks 3 and 6). In all instances, the identity of the milk proteins was confirmed by antibody precipitation (results not shown).

Although all the milk proteins were synthesized by the 10 h and 72 h post-partum gland, it was apparent that in the 72 h post-partum tissue, casein C was particularly predominant, whereas α -lact-

Fig. 2. Analysis of milk protein synthesis by explants of mammary tissue in culture, taken from guinea-pigs at parturition or 10 h and 72 h post-partum

[^{35}S]Methionine-labelled polypeptides were analysed as described in Fig. 2. Samples were as follows: total [^{35}S]labelled polypeptides present in tissue (tracks 1, 2 and 3) or secreted into medium (tracks 4, 5 and 6). Tracks 1 and 4, parturition tissue; tracks 2 and 5, 10 h post-partum; tracks 3 and 6, 72 h post-partum.

albumin predominated in the secretions (Fig. 2, compare tracks 3 and 6). In addition, multiple bands were present in the caseins A, B, and C regions of the gel. These were especially prominent in the 72 h post-partum tissue (Fig. 2, track 3). This heterogeneity probably reflects various stages of modification of the polypeptides, in particular phosphorylation (J. C. Pascall & A. P. Boulton, unpublished results).

It was apparent from the results described above that the abundant cDNA hybridization probe was not sufficiently enriched with α -lactalbumin cDNA to provide meaningful analysis of the pregnant tissue, as examination of the spectrum of proteins synthesized by explants using antibody precipitation indicated that α -lactalbumin was being synthesized. As a means of quantifying the amounts of individual milk protein RNA transcripts present at different stages of mammary development, we have purified ^3H -labelled DNA complementary to the individual mRNA species for α -lactalbumin and caseins A, B and C from a cDNA population synthesized from the total mammary gland post-nuclear poly(A)-containing RNA population of a lactating animal. This was carried out by hybridization to recombinant plasmid DNA immobilized on diazobenzylmethyl-paper under stringent conditions (see the Materials

Fig. 3. Comparison of the relative amounts of caseins A, B, and C RNA transcripts within the guinea-pig mammary gland during mammary development pre- and post-partum

Total RNA isolated from guinea-pig mammary tissue from animals 62 days pregnant (○), in premature labour (△), at parturition (□), and 8 h (●) and 7 days (■) post-partum were hybridized in RNA excess to sequence-specific cDNA hybridization probes at RNA concentrations of 50 µg/ml, 1 mg/ml and 2.5 mg/ml. Line fitting of these and all subsequent curves was by computer. R_0t values represent the product of RNA concentration (mol of nucleotide/litre) and reaction time(s). The extent of hybridization represents the percentage of cDNA resistant to S_1 nuclease at the end of the reaction. (a) Casein A cDNA probe; (b) casein B cDNA probe; (c) casein C cDNA probe.

and methods section). For these experiments recombinant plasmids pgp K-9, pgp N-33, pgp K-27, and pgp O-22, containing DNA sequences complementary to the mRNAs for α -lactalbumin and caseins A, B, and C respectively were used (Craig *et al.*, 1981). Individual milk protein cDNA sequences purified in this manner, when back-hybridized to a post-nuclear poly(A)-containing RNA population from a 7 day post-partum lactating guinea-pig mammary gland, all hybridized as a single component with the expected kinetics, that of an abundant mRNA sequence (results not shown).

Analysis of total mammary RNA from 62 day pregnant animals using specific cDNA probes for the mRNAs for caseins A, B, and C (Fig. 3), showed that there was incomplete protection of each hybridization probe by RNA from the pregnant animal (Figs. 3a, 3b and 3c) an indication of the absence of significant amounts of these sequences. Full protection was observed by using RNA isolated from animals in premature labour at parturition, and RNA from animals 8 h and 7 day post-partum. In all instances, the relative concentration of the individual mRNA sequences in tissue from animals at parturition was almost indistinguishable from tissue from animals 8 h or 7 day post-partum, whereas the

Fig. 4. Comparison of the relative amounts of α -lactalbumin RNA transcripts within the guinea-pig mammary gland during mammary development pre- and post-partum

Total RNA isolated from guinea-pig mammary tissue from animals 62 day pregnant (○), in premature labour (△), at parturition (□), and 8 h (●) and 7 days (■) post-partum were hybridized in RNA excess to an α -lactalbumin sequence-specific cDNA probe essentially as described in the legend to Fig. 3.

relative concentration of each mRNA sequence in RNA from the tissue from animals in premature labour varied from 5–6-fold lower for caseins A and B to 9-fold lower for casein C.

Analysis of the relative concentration of α -lactalbumin mRNA sequences in the different RNA populations identified the presence of α -lactalbumin transcripts in the tissue from 62 day pregnant animals (Fig. 4), and showed that, between this stage of development and parturition, there was a 100-fold increase in the relative concentration of this sequence. Surprisingly, after parturition there was a small but significant decrease in the relative concentration of α -lactalbumin sequences by 8 h post-partum (2-fold) and a further slight decrease by 7 days post-partum. Analysis of the tissue from animals in premature labour showed that the relative concentration of α -lactalbumin sequences was the same as at 7 days post-partum, in contrast to the relative concentration of casein mRNA sequences at the same time points (see Fig. 3).

Estimation of the number of each individual mRNA sequence present per cell at the different stages of development was not attempted. Accurate numbers may only be assessed when the proportion of cells within the mammary tissue that are transcribing milk protein genes at different times during development has been determined.

Discussion

In the guinea-pig, the most rapid period of mammary development occurs during and after parturition (Nelson *et al.*, 1962). We demonstrate here that differential expression of α -lactalbumin and casein gene expression occurs during the onset of lactation in the guinea-pig mammary gland, and that milk protein synthesis and secretion occur throughout the major mammary development period after parturition.

We have previously established a major role for the post-transcriptional regulation of gene expression in the lactating guinea-pig mammary gland (Bathurst *et al.*, 1980*a,b*), and have surmised, on the basis of the relative abundance of the milk protein mRNA sequences in the nuclear and post-nuclear fractions of the lactating mammary gland, that transcriptional mechanisms must be involved (Bathurst *et al.*, 1980*b*; Craig *et al.*, 1980). Our studies here support these views. The massive accumulation of casein RNA transcripts during the last 48 h of pregnancy, and to a lesser extent the accumulation of α -lactalbumin mRNA transcripts, must be due in part to the elevated levels of transcription. It is likely that post-transcriptional mechanisms also operate, as it has been demonstrated in studies on casein mRNA synthesis in the

rat mammary gland (Guyette *et al.*, 1979) that preferential stabilization of the milk protein mRNA contributes significantly to the rapid accumulation of these sequences.

Translational control mechanisms also operate and regulate the onset of casein synthesis at parturition. At this phase of mammary development, the relative concentration of casein mRNA was already maximal, but only α -lactalbumin synthesis and secretion could be detected in organ culture. As in the lactating gland only 2–3% of milk protein RNA transcripts are located in the nucleus (Bathurst *et al.*, 1980*b*), it seems likely that at parturition the bulk of the casein gene transcripts [we estimate over 50 000 copies of each casein sequence per cell (see Bathurst *et al.*, 1980*b*)] will be located in the cytoplasm, possibly in the form of messenger ribonucleoprotein particles. This observation is interesting when compared with studies on the hormonal regulation of hepatic α_{2u} globulin (Kurtz *et al.*, 1978). In this instance transcriptional control was exerted by steroids, but the initiation of protein synthesis was dependent on the presence of growth hormone.

In addition to providing evidence for translational control, studies on the synthesis of the caseins and α -lactalbumin by explants of mammary tissue in organ culture provide some interesting insights into the kinetics of milk protein secretion. These show that different milk proteins are secreted at different rates within the same tissue. It is clear, for instance, that in tissue from animals 72 h post-partum (Fig. 3) α -lactalbumin is secreted most rapidly, whereas caseins A and B are secreted faster than casein C. Previous studies on the rates of secretion of milk proteins employed oocytes of *Xenopus laevis* as a surrogate system with which to study the mechanisms of secretion in living cells. Microinjection of milk protein mRNA into oocytes results in the sequestration and subsequent secretion of α -lactalbumin and the caseins, though the latter are not correctly modified (see Colman & Morser, 1979; Lane *et al.*, 1980). In these studies, caseins A and B were also secreted more rapidly than casein C, but unlike the situation in the mammary gland, α -lactalbumin was secreted more slowly than the caseins.

At present we have little knowledge of the intracellular mechanisms which regulate the rate of secretion of individual polypeptides, though clearly they exist. The comparative studies of casein secretion by the oocyte and mammary gland do, however, provide some insight. In the negative sense, post-translational modifications are not a prerequisite for secretion (see Colman *et al.*, 1981), and in the positive sense the rate of secretion seems to be inherently determined by the primary structure of the polypeptide (or at least the conformation it takes

up within the secretory apparatus). If this were not the case it would be difficult to rationalize why secretion by the oocyte of the foreign casein polypeptides should mimic the differential rates of secretion observed within the mammary gland. Unfortunately, the same argument cannot be applied to α -lactalbumin. Should it prove that α -lactalbumin is synthesized in a different cell from the caseins, then the comparison of the relative rates of secretion of α -lactalbumin and the caseins after micro-injection of total milk protein mRNA into the oocyte becomes less meaningful.

This and other studies (Bathurst *et al.*, 1980a,b; Craig *et al.*, 1980) demonstrate that in the guinea-pig mammary gland the mechanisms involved in milk protein gene expression are highly complex and operate at not one, but many, intracellular levels. Why α -lactalbumin mRNA concentrations should be maximal at parturition remains obscure. It is possible that this reflects a very early nutritional requirement of the young. Consequently, as suggested by Brew & Hill (1975), the synthesis of α -lactalbumin late in pregnancy reflects a gland primed to synthesize lactose, but that maximal synthesis does not occur until the commencement of suckling, presumably as a component of the colostrum. A similar argument would equally account for the accumulation of casein mRNA in anticipation of normal milk synthesis, at some point after parturition.

Studies using explants of mouse mammary gland in organ culture also reveal the differential regulation of α -lactalbumin and casein genes (Ono & Oka, 1980a,b). Evidence for a similar differential expression of the milk protein genes in other species requires clarification. In the rat, as in the mouse, mammary development occurs throughout pregnancy (see Anderson, 1974). During this period casein mRNA is present in significant amounts (Rosen *et al.*, 1978; Nardacci *et al.*, 1978; Nakhasi & Qasba, 1979), as is α -lactalbumin mRNA (Nakhasi & Qasba, 1979), though in one instance α -lactalbumin mRNA (assayed by cell-free protein synthesis) was found only in significant amounts at the onset of lactation (Nardacci *et al.*, 1978).

Overall, evidence accumulated from several different species shows that the regulation of milk protein gene expression is more intricate than was previously envisaged.

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