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Characterization of the translation products of the major mRNA species from rabbit lactating mammary glands and construction of bacterial recombinants containing casein and α-lactalbumin complementary DNA

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Total cytoplasmic polyadenylated RNA from lactating rabbit mammary glands was analysed on methylmercury hydroxide–agarose gels. The size of the most abundant mRNA species ranged between 0.5 and 5.0kb (kilobases), with major bands at 0.55, 0.84, 0.92, 1.18 and 2.4kb and discrete minor bands of 1.5, 1.7, 3.0 and 3.9kb. Translation *in vitro* of total mRNA with [^{3}H]leucine or [^{35}S]methionine as precursor yielded four major bands with apparent M_r values of 16000, 25000, 26000 and 29000. The four protein bands were identified by immunoprecipitation by using specific antisera as α-lactalbumin and x-, κ- and α-caseins, respectively. Labelling with [^{35}S]cysteine followed by immunoprecipitation with anti-transferrin or anti-α-lactalbumin sera allowed the identification of two whey proteins. Translated transferrin was resolved as an 80000-dalton band and α-lactalbumin appeared as a 16000-dalton protein. A library of recombinant plasmids containing cDNA (complementary DNA) sequences representing cytoplasmic polyadenylated RNA was used to isolate clones for the major rabbit caseins and α-lactalbumin. A preliminary characterization of these cDNA clones was achieved by colony hybridization with enriched RNA fractions as probes. Positive clones were identified by use of hybrid-promoted translation *in vitro* and immunoprecipitation of the translation products. The corresponding mRNA species were further identified by hybridizing RNA blots with radioactively labelled cDNA clones. We present the restriction map of α-casein and κ-casein cDNA clones.

The major function of the mammary gland is to synthesize and secrete large amounts of milk proteins. Whereas some milk proteins are derived from blood, e.g. albumin and immunoglobulin G, most proteins are produced in the mammary gland. The biosynthesis of caseins and α-lactalbumin and the mechanisms involved in the post-translational modifications and secretion of these proteins have been extensively studied, mostly in the guinea pig (Craig *et al.*, 1978, 1979*a*,*b*; Pascall *et al.*, 1981). Like most secretory proteins, milk proteins are synthesized as precursors with transient *N*-terminal sequences, which are subsequently cleaved by membrane-bound signal peptidases (Blobel & Dobberstein, 1975*a*,*b*), as demonstrated for rat (Lingappa *et al.*, 1978), ewe (Gaye *et al.*, 1979) and guinea pig (Craig *et al.*, 1976) α-lactalbumin and rat (Rosen & Shields, 1980), ewe (Gaye *et al.*, 1977) and guinea-pig (Zehavi-Willner & Lane, 1977; Craig *et al.*, 1979*a*; Pascall *et al.*, 1981) caseins. Such proteolytic cleavages and co-translational core-glycosylation of milk proteins have been shown to result in a change in mobility of peptides when analysed on SDS/polyacrylamide-gel electrophoresis compared with their counterpart synthesized in cell-free translation systems in the absence of heterologous microsomal membranes (Craig *et al.*, 1979*a*,*b*).

It is now well established in the rabbit (Houdebine & Gaye, 1975; Houdebine, 1976; Devinoy *et al.*, 1978; Houdebine *et al.*, 1978), the rat (Rosen & Barker, 1976; Guyette *et al.*, 1979; Rosen *et al.*, 1978, 1980) and the mouse (Banerjee *et al.*, 1978; Terry *et al.*, 1977) that the expression of casein and α-lactalbumin genes is hormonally regulated. In all

Abbreviations used: SDS, sodium dodecyl sulphate; cDNA, complementary DNA; poly(A)$^+$ RNA, polyadenylated RNA; MeHgOH, methylmercury hydroxide; kb, kilobases; DBM-, diazobenzyloxymethyl-.

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these species, lactogenic hormones (prolactin, choriomammotropin and primate somatotropin) appear to be the primary regulators of expression of genes for caseins and α-lactalbumin (Matusik & Rosen, 1978; Craig & Campbell, 1978; Burditt *et al.*, 1981).

Isolation of mRNA species for milk proteins, subsequent cDNA synthesis and cloning should provide biochemical probes that are useful to quantify mRNA induction in response to various hormonal stimuli. Since it has already been shown that synthesis and secretion of milk proteins are differently regulated by hormones (Nardacci *et al.*, 1978; Häuptle *et al.*, 1979; Ono & Oka, 1980), it would be of interest to prepare cDNA probes for the various milk proteins in order to test whether control occurs at the transcriptional level exclusively, or whether post-transcriptional, translational or post-translational events affect the rates of synthesis of the various milk proteins. In rat mammary-organ culture prolactin induces an accumulation of casein mRNA by increasing casein-mRNA synthesis approx. 4-fold and extending the half-life of casein mRNA approx. 20-fold (Guyette *et al.*, 1979).

Despite extensive work, the molecular mechanism whereby lactogenic hormones act on gene expression remains unknown. Attempts to identify possible mediators have thus far failed (Matusik & Rosen, 1980). We have shown that lactogenic hormones are rapidly internalized in dispersed mammary cells, with subsequent translocation to the nucleus as evidenced by electron-microscope radioautography (Suard *et al.*, 1979). To test whether such a nuclear translocation process has any physiological significance, specific cDNA clones are needed as hybridization probes.

In the present paper we report the characterization of the translation products of six milk-protein mRNA molecules and the construction of bacterial recombinants containing cDNA for α- and κ-casein and α-lactalbumin.

Materials and methods

Materials

Animals. From New Zealand white outbred rabbits, 4-day-lactating mammary glands were removed.

Reagents. The following reagents were used: oligo(dT)–cellulose (type II) and $(dT)_{12-18}$ from Collaborative Research; poly(U) from Miles Laboratories; MeHgOH (water-soluble) from Alpha; protein A–Sepharose from Pharmacia; calf thymus terminal transferase (specific activity 3500 units/mg) and DNA polymerase I (specific activity 3500 units/mg) from Boehringer; T4 polynucleotide kinase (specific activity 31000 units/mg) from P-L Biochemicals; tetracycline hydrochloride (Achromycin) from Cyanamid G.m.b.H., Wolfrathausen, Germany; [^{3}H]leucine (100 Ci/mmol) from New England Nuclear; [^{35}S]methionine (>1100 Ci/mmol), [^{35}S]cysteine (>440 Ci/mmol), [α-^{32}P]dCTP (>400 Ci/mmol) and [γ-^{32}P]ATP (>2000 Ci/mmol) from The Radiochemical Centre.

Reverse transcriptase from avian myeloblastosis virus was a gift from Dr. J. Beard (National Cancer Institute, Bethesda, MD, U.S.A.), and S1 nuclease was from Dr. U. Schibler (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland). Restriction enzymes were purchased from Biolabs, except *Eco*RI, obtained from Boehringer, and *Pst*I, generously given by Dr. G. Fey (Swiss Institute for Experimental Cancer Research). All the reagents were of analytical grade. Phenol was redistilled before use, formamide was recrystallized twice, and *Escherichia coli* tRNA (Sigma) was extracted with phenol/chloroform (1:1, v/v) before use.

Methods

Poly(A)$^+$ RNA isolation. Cytoplasmic RNA from 4-day-lactating rabbit mammary gland was extracted as described by Schibler *et al.* (1980). Poly(A)$^+$ RNA was isolated by two passages of heat-denatured cytoplasmic RNA over oligo(dT)–cellulose.

mRNA analysis on denaturing agarose gels. Electrophoresis of poly(A)$^+$ RNA in 2% agarose gel containing 5 mM-MeHgOH was performed as described by Bailey & Davidson (1976). The size of the mRNA species was determined by 18S and 28S mammary-gland rRNA, *E. coli* 16S and 23S rRNA and mouse pancreas mRNA of known size (1.74, 0.92 and 0.84 kb; Schibler *et al.*, 1980) as standards.

Separation of mRNA species by sucrose-density-gradient centrifugation. Poly(A)$^+$ RNA (60–100 μg) was heat-denaturated and layered on a 5–25% (w/w) linear sucrose gradient containing 0.5% SDS in 10 mM-Tris/HCl, pH 7.4. The gradients were centrifuged at 23000 $g_{av.}$ for 16 h in a Beckman SW 40 rotor. Fractions (400 μl) were collected and the mRNA activity was assessed by cell-free translation as described below.

Cell-free protein synthesis and product analysis. Cell-free translation was performed with a commercial rabbit reticulocyte lysate (New England Nuclear) with [^{3}H]leucine, [^{35}S]methionine or [^{35}S]-cysteine as radioactive precursors. Translation conditions were as specified by the supplier and essentially as previously described (Pelham & Jackson, 1976). Total protein synthesis was determined by precipitation with hot trichloroacetic acid (Gordon *et al.*, 1977) and portions were resolved by SDS/polyacrylamide-gel electrophoresis (in 10%

gels) followed by fluorography. Characterization of the polypeptides synthesized by the cell-free system was performed by indirect immunoprecipitation. Specific antisera prepared against α-, κ- or x-casein, α-lactalbumin and milk transferrin (Dayal *et al.*, 1982) were used, and protein A–Sepharose served to precipitate antigen–antibody complexes as described by Mostov *et al.* (1980). The labelled peptides were analysed by SDS/polyacrylamide-gel electrophoresis (in 7.5–15% gradient gels), unless otherwise stated, and revealed by fluorography with Kodak X-O-mat S films and amplifying screens (Laskey & Mills, 1975).

Molecular cloning of cDNA. Double-stranded cDNA was synthesized as described by Schibler *et al.* (1980). The double-stranded cDNA made against 50 μg of poly(A)$^+$ RNA was size-fractionated (0.5–1.2 kb) on a neutral 2% agarose gel alongside endonuclease-*Hinf* I restriction fragments of plasmid pBR322 as size markers. The size-selected double-stranded cDNA was 'tailed' with an average of 10 dCTP residues by using terminal deoxynucleotidyl transferase as described by Schibler *et al.* (1980). The vector pBR322, cleaved with *Pst*I and 'tailed' with dG to a length of 10 bases, was generously supplied by Dr. U. Schibler. An equimolar mixture of the tailed double-stranded cDNA and plasmid was annealed as described by Schibler *et al.* (1980). The DNA preparation was then used for transformation of *E. coli* strain HB101 as described by Wensink *et al.* (1974).

Colony hybridization. This was done essentially as described by Grunstein & Hogness (1975). For the selection of casein-cDNA clones, we used a fraction of a preparative sucrose gradient highly enriched in casein mRNA as evidenced by its translation activity in the cell-free system. For the screening of α-lactalbumin-cDNA clones, the 0.55 kb-mRNA species, eluted from a MeHgOH–agarose gel and shown to direct the synthesis of α-lactalbumin, was used as specific probe. The α-lactalbumin-mRNA band was excised and passed through a no. 27-gauge needle, and RNA was recovered from the gel slices as described by Schibler *et al.* (1980), except that ammonium acetate was used instead of LiCl to neutralize MeHgOH.

mRNA molecules to be used as probes were treated with 0.1 M-NaOH for 1 h at 4°C, neutralized, precipitated with ethanol, and end-labelled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Maxam & Gilbert, 1977). A specific radioactivity of 2.5×10^7 c.p.m./μg was routinely obtained.

Isolation of plasmid DNA. Individual colonies were isolated and grown in suspension culture, plasmids were amplified with chloramphenicol (0.2 mg/ml), and a cleared lysate was prepared (Katz *et al.*, 1977). Plasmid DNA was purified by banding in a CsCl gradient containing ethidium bromide as described previously (Clewell & Helinski, 1972).

Gel electrophoresis and elution of DNA. Digestion of recombinant DNA molecules with restriction enzymes was performed according to the instructions furnished by the supplier. Restriction fragments were separated on horizontal 2% agarose gels (McDonnell *et al.*, 1977) and DNA bands were detected by staining the gels with ethidium bromide in electrophoresis buffer.

Preparative elution of DNA fragments from low-melt agarose was performed as described by McMaster *et al.* (1981).

Nucleic acid blotting techniques and hybridization. DNA fragments were transferred from agarose gels to nitrocellulose sheets as described by Southern (1975). Immobilized DNA was hybridized by using recombinant-DNA probes after nick-translation labelling (Maniatis *et al.*, 1975).

RNA electrophoresed on MeHgOH–agarose gels was transferred to DBM-paper and hybridized with labelled DNA probes as described by Alwine *et al.* (1977).

Hybrid-promoted translation. Linearized plasmids containing cDNA inserts were immobilized on DBM-paper and hybridized with total poly(A)$^+$ RNA as described by Stark & Williams (1979). After elution, RNA was translated and immunoprecipitated with specific antisera.

Restriction-enzyme mapping. Restriction-endonuclease cleavage sites were mapped by the method of Smith & Birnstiel (1976). The orientation of the cDNA clones was determined by hybridizing a Southern blot containing the appropriate restriction-endonuclease fragments with [γ-^{32}P]ATP-end-labelled poly(U). Hybridization conditions were as described by Schibler *et al.* (1980), except that carrier DNA was replaced by *E. coli* tRNA (10 μg/ml) and the hybridization temperature was 37°C.

Containment. All work involving recombinant DNA was carried out in a P3 facility by using the EKI biological containment (*E. coli* HB101, plasmid pBR322), according to the National Institutes of Health guidelines of 21 November 1980 (Federal Register, 1980).

Results

Characterization of poly(A)$^+$ mRNA

Cytoplasmic poly(A)$^+$ RNA was isolated from 4-day-lactating rabbit mammary glands and purified by affinity chromatography. After two passages on oligo(dT)–Sepharose, the poly(A)$^+$ RNA fraction represented 0.7–1.3% of the total RNA. The recovery was approx. 15 μg of poly(A)$^+$ RNA/g of tissue. The size of the most abundant mRNA species, as determined by MeHgOH–agarose-gel electrophoresis (Fig. 5), ranged between 0.55 and

5.0kb, with major bands at 0.55, 0.84, 0.92, 1.18 and 2.4kb and minor discrete bands at 1.5, 1.7, 3.0 and 3.9kb. The cytoplasmic poly(A)$^+$ RNA preparation was slightly contaminated by rRNA species.

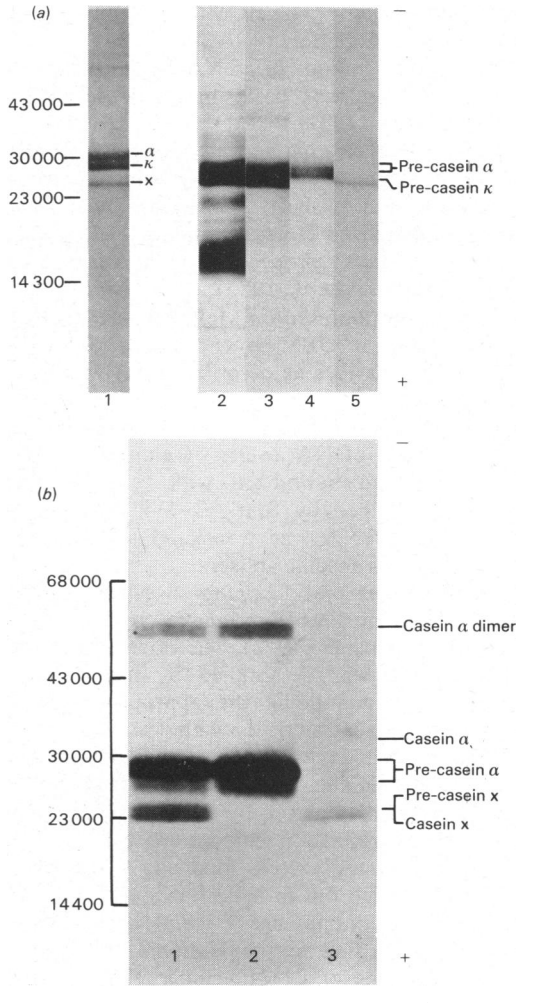

Fig. 1. *Immunoprecipitation of translation products with anti-casein sera*

(*a*) The [^{35}S]methionine-labelled translation products were analysed by SDS/polyacrylamide-gel electrophoresis (13% gels). Lanes: 1, Coomassie Blue staining of purified rabbit milk caseins; 2, total translated [^{35}S]methionine-labelled proteins; 3, precipitation with anti-(whole casein) serum; 4, precipitation with anti-(casein α) serum; 5, precipitation with anti-(casein κ) serum. (*b*) The [^{3}H]-leucine-labelled translation products were analysed by SDS/polyacrylamide-gel electrophoresis (10% gels). Lanes: 1, precipitation with anti-(whole casein) serum; 2, precipitation with anti-(casein α) serum; 3, precipitation with anti-(casein x) serum.

Identification of translated milk proteins

Polypeptides specified by lactating-mammary-gland mRNA molecules were identified by cell-free translation of cytoplasmic poly(A)$^+$ RNA, followed by immunoprecipitation with antisera directed against α-, x- and κ-caseins, α-lactalbumin or transferrin. As expected from the amino acid composition of each milk protein, various extents of radioactivity incorporation in individual translated peptides were achieved with the three radioactive amino acids used as precursors. Thus α- and κ-caseins incorporated [^{3}H]leucine and [^{35}S]-methionine preferentially, transferrin [^{3}H]leucine and [^{35}S]cysteine, x-casein mainly [^{3}H]leucine, and α-lactalbumin all three precursors equally. The immunoprecipitated translated products were analysed by SDS/polyacrylamide-gel electrophoresis (Figs. 1 and 2) and the apparent M_r values estimated from their electrophoretic mobilities were compared with those of the corresponding secreted polypeptides (Table 1).

α-Pre-casein was resolved as a doublet of 29000 and 27000 daltons, i.e. with smaller M_r than the secreted α-casein (31000); x-pre-casein, with M_r 25000, co-migrated with the mature form; κ-pre-casein appeared as a single band (26000 daltons) migrating faster than the secreted protein (29000 daltons). Although each anti-casein serum was monospecific, cross-contamination could occur dur-

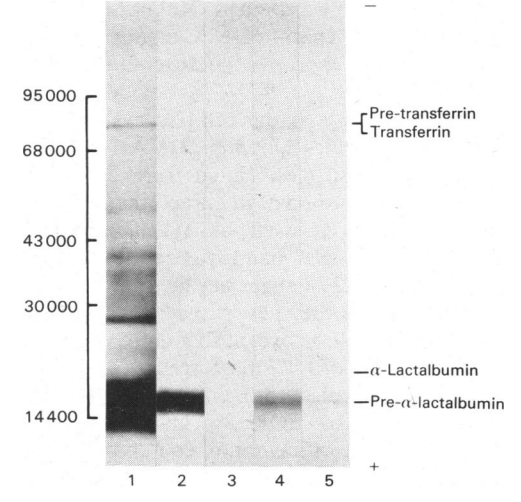

Fig. 2. *Immunoprecipitation of [^{35}S]cysteine-labelled translation products with antisera directed against specific whey proteins*

Lanes: 1, total [^{35}S]cysteine-labelled translation products; 2, precipitation with anti-(α-lactalbumin) serum (M_r 16000); 4, precipitation with anti-transferrin serum (M_r 80000); 3 and 5, controls using pre-immune sera.

Table 1. *Apparent M_r values of translated and of secreted milk proteins*
For experimental details see the text.

Protein	Radioactive precursor	M_r Translated	M_r Secreted
Casein α	[^{3}H]Leucine, [^{35}S]methionine	29000, 27000	31000
Casein κ	[^{3}H]Leucine, [^{35}S]methionine	26000	29000
Casein x	[^{3}H]Leucine	25000	25000
α-Lactalbumin	[^{3}H]Leucine, [^{35}S]methionine, [^{35}S]cysteine	16000	22000
Milk transferrin	[^{3}H]Leucine, [^{35}S]cysteine	80000	80000

ing immunoprecipitation, probably owing to the hydrophobic nature and micellar configuration of caseins, unless immunoprecipitation was carried out under conditions including a heating step in the presence of 2% SDS followed by a 5-fold dilution with buffer containing Triton X-100 before incubation with antiserum (Mostov *et al.*, 1980). Immunoprecipitation with anti-(whole casein) or anti-(α-casein) sera in the absence of EDTA from the incubation buffer resulted in an additional band, the M_r of which was consistent with that of a α-pre-casein dimer (Fig. 1*b*). No polymerization of κ- or x-pre-caseins was observed in the absence of EDTA. Pre-α-lactalbumin (16000 daltons) migrated much faster than the secreted glycoprotein, and no change of mobility was observed between pre-transferrin and secreted transferrin.

Construction of a double-stranded-cDNA library

The experimental protocol for the cloning of the three major rabbit milk-protein mRNA species consisted of the synthesis of double-stranded cDNA from 50μg of poly(A)$^+$ RNA of 4-day-lactating mammary glands by using avian-myeloblastosis-virus reverse transcriptase. After S1-nuclease digestion, double-stranded cDNA species of between 0.5 and 1.2kb were recovered from a preparative neutral agarose gel, tailed with an average of 10dC residues and annealed in a 1:1 molar ratio with *Pst*I-cleaved and dG-tailed pBR322 plasmid. Upon transformation of the *E. coli* strain HB101, 7 colonies were obtained per ng of plasmid, corresponding to a total of 480 tetracycline-resistant transformants.

Identification of casein and α-lactalbumin cDNA clones

For colony hybridization, poly(A)$^+$ RNA was sedimented by SDS/sucrose-density-gradient centrifugation, and the fractions enriched in casein mRNA and devoid of α-lactalbumin-mRNA activity were used as ^{32}P-labelled probes. α-Lactalbumin mRNA eluted from preparative MeHgOH–agarose gels and ^{32}P-end-labelled served as a screening probe for α-lactalbumin-cDNA clones. The DNA of 15 colonies out of 200 hybridized preferentially with casein mRNA, and 10 colonies out of 100 gave a more intense hybridization signal with α-lactalbumin mRNA. The colonies giving the strongest signals were amplified, and plasmids isolated from each positive clone were analysed for insert length on a 2% neutral agarose gel after *Pst*I digestion. The casein-cDNA clones with the largest insertions contained cDNA sequences varying from 680 (clone designated pRcas27) to 750 base-pairs (clone designated pRcas71). The length of the α-lactalbumin insertion was estimated at 530 base-pairs for two clones designated pRlac1 and pRlac7. By using *Hinf*I, two different restriction patterns were observed for the casein-cDNA clones pRcas27 and pRcas71, indicating the presence of at least two different casein-cDNA sequences (results not shown).

Hybridization of mammary-gland poly(A)$^+$ RNA to DBM-paper filters containing cloned α-lactalbumin- or casein-cDNA sequences was used for the final characterization of each of the clones. mRNA eluted from each filter was translated in the cell-free system; the translated products were immunoprecipitated with the appropriate antiserum and finally resolved on a 7.5–15% gradient gel by SDS/polyacrylamide-gel electrophoresis. The results of such an experiment are illustrated in Fig. 3. mRNA eluted from clone pRcas27 was translated into a product co-migrating with casein κ (lane 2) and precipitated exclusively with anti-(casein κ) serum (lane 4). Thus this clone will be termed pRcasκ27. With clone pRcas71 a mRNA was selected which specified casein α (lane 5), as demonstrated by immunoprecipitation of its translation product *in vitro* (lane 6). This clone will be referred to as pRcasα71. In lane 6, two bands were observed, the upper doublet band presumably generating the lower band by proteolytic degradation. Hybrid-promoted translation of the smaller rabbit milk protein, α-lactalbumin, is shown in Fig. 4. The mRNA eluted from hybrids with both clones pRlac1 and pRlac7 yielded on translation a major 16000-dalton band identified as α-lactalbumin by using anti-(α-lactalbumin) serum.

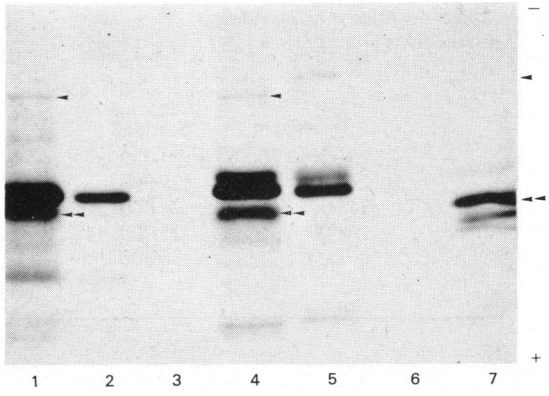

Fig. 3. *Hybrid-promoted translation of pRcas 27 and pRcas 71 cDNA clones*

Translation products were analysed by SDS/polyacrylamide-gel electrophoresis (7.5–15% gradient gels). Lanes 1–3, translation of mRNA eluted from clone pRcas 27. Lanes 4–6, translation of mRNA eluted from clone pRcas 71. Lanes 1 and 4, total translation of eluted mRNA. Lanes 3 and 5, immunoprecipitation with anti-(casein α) serum. Lanes 2 and 6, immunoprecipitation with anti-(casein κ serum). Lane 7, endogenous activity of the reticulocyte lysate (marked by arrow-heads).

Identification of the mRNA species for α-casein, κ-casein and α-lactalbumin

Clones containing cDNA sequences encoding α-casein, κ-casein or α-lactalbumin were used to determine the size of the corresponding mRNA molecules. Each plasmid was nick-translated with [α-^{32}P]dCTP and hybridized to mammary-gland poly(A)$^+$ RNA which had been electrophoresed on a 2% MeHgOH–agarose gel and transferred to DBM-paper filters. The results obtained for two casein-cDNA and two α-lactalbumin-cDNA clones are illustrated in Fig. 5. Both α-lactalbumin-cDNA clones hybridized to the 0.55 kb-RNA species (lanes 4 and 5). The two casein-cDNA clones (pRcasκ27 and pRcasα71) hybridized exclusively to the large 1.18 kb-RNA band (lanes 2 and 3). Since pRcasκ27 and pRcasα71 encode different caseins (Fig. 3), this finding indicates that the 1.18 kb-RNA band is heterogeneous and contains at least two mRNA populations of similar size.

Comparison of the cDNA insertion length with the size of the respective milk-protein mRNA estimated by MeHgOH–agarose-gel electrophoresis indicates that the α-lactalbumin-cDNA clones are almost full length, whereas the casein-cDNA clones contain 60% (pRcasκ27) or 65% (pRcasα71) of the mRNA sequence.

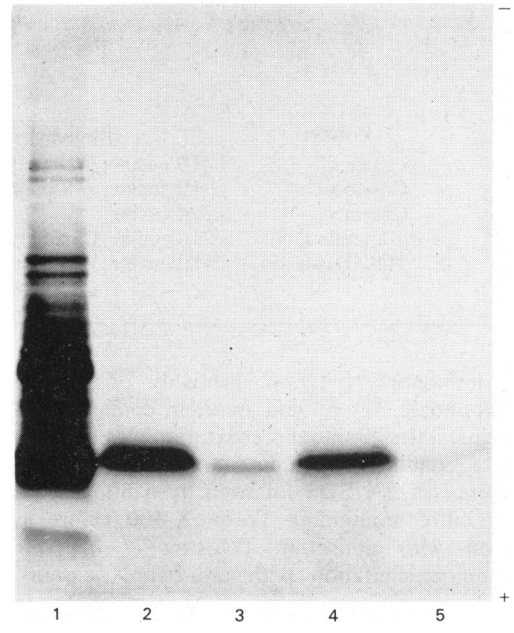

Fig. 4. *Hybrid-promoted translation of pRlac1 and pRlac7 cDNA clones*

Translation products were analysed by SDS/polyacrylamide-gel electrophoresis (7.5–15% gradient gels). Lane 1, [^{35}S]methionine-labelled translation products of total poly(A$^+$) RNA. Lanes 2 and 3, translation of mRNA eluted from clone pRlac1. Lanes 4 and 5, translation of mRNA eluted from clone pRlac7. Lanes 2 and 4, total translation of eluted mRNA. Lanes 3 and 5, immunoprecipitation with anti-(α-lactalbumin) serum.

Restriction-enzyme mapping and comparison of the casein-cDNA clones

The restriction map of the pRcasκ27 and pRcasα71 clones obtained by partial restriction-enzyme digestion (Smith & Birnstiel, 1976) is given in Fig. 6. The following restriction enzymes were tested: *Eco*RI, *Eco*RII, *Hae*III, *Hha*I, *Hind*III, *Hpa*II, *Pst*I, *Sau*3AI, *Taq*I.

The orientation of the α- and κ-casein-cDNA sequences was determined by using ^{32}P-labelled poly(U) as a probe for the 3′ end of the cDNA. This probe was hybridized to 'Southern' blots containing restriction fragments of each cDNA clone. The 240-base-pair *Eco*RII fragment of the pRcasα71 clone and 130-base-pair *Hinf*I fragment of the pRcasκ27 clone strongly hybridized with the poly(U) probe (Fig. 6). The specificity of hybridization was assessed by the absence of hybridization with the *Hinf*I fragments of the vector pBR322 present in the blot (results not shown). These results demonstrate

that each casein clone contains a stretch of A residues representing at least a portion of the mRNA poly(A) tail. Therefore the two cDNA restriction maps can be aligned and compared. As shown in Fig. 6, there is no apparent homology among the restriction maps. This is consistent with the results obtained by hybrid-promoted translation, in which no cross-hybridization was detected between the two casein-mRNA species. However, the existence of small regions of homology cannot be ruled out.

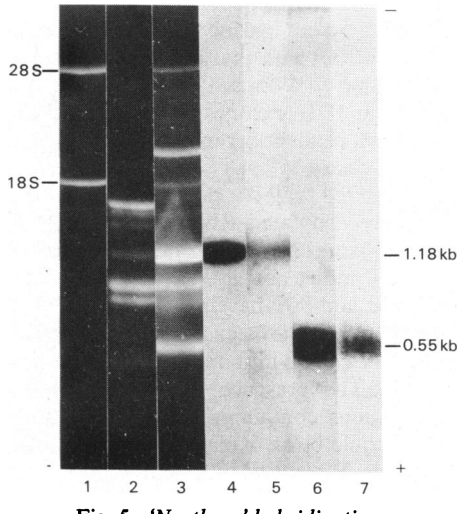

Fig. 5. *'Northern' hybridization*
Cytoplasmic poly(A)$^+$ RNA (lane 3) was electrophoresed on 2% MeHgOH–agarose gels alongside mammary-gland 18 and 28S rRNA markers (lane 1) and mouse pancreas poly(A)$^+$ RNA (lane 2; the sizes of the most abundant species are 1.74, 0.92 and 0.84kb). The gel was stained with ethidium bromide. After electrophoresis, the poly(A)$^+$ RNA was transferred to DBM-paper (lanes 4–7) and hybridized with nick-transi ted clones pRcasκ27 (lane 4), pRcasα71 (lane 5), pΓ!ac1 (lane 6) and pRlac7 (lane 7).

Discussion

Rabbit milk separated by isoelectric precipitation and analysed by SDS/polyacrylamide-gel electrophoresis contains four major proteins, three acid-precipitable caseins and one whey protein termed α-lactalbumin (Dayal *et al.*, 1982). In addition, two less abundant whey proteins, transferrin and secretory component, were also identified and shown to be synthesized in the rabbit mammary gland (Dayal *et al.*, 1982; Mostov *et al.*, 1980). The three rabbit caseins are rich in glutamic acid, valine, proline and leucine, and exhibit variable degrees of glycosylation and phosphorylation, properties which they share with bovine (Jollès, 1972), human (Nagasawa *et al.*, 1967) and rat caseins (Nardacci *et al.*, 1978). By analogy with the bovine system, we termed the rabbit caseins α-casein (M_r 31000) β-casein (M_r 29000), κ-casein (M_r 29000) and x-casein (M_r 25000) (Dayal *et al.*, 1982; Mercier & Gaye, 1980).

Species of mRNA coding for milk proteins have been isolated and characterized from the rat (Rosen *et al.*, 1975), the ewe (Houdebine & Gaye, 1975) and the guinea pig (Craig *et al.*, 1976). Cytoplasmic poly(A)$^+$ RNA isolated from 4-day-lactating rabbit mammary glands was resolved by MeHgOH–aga-

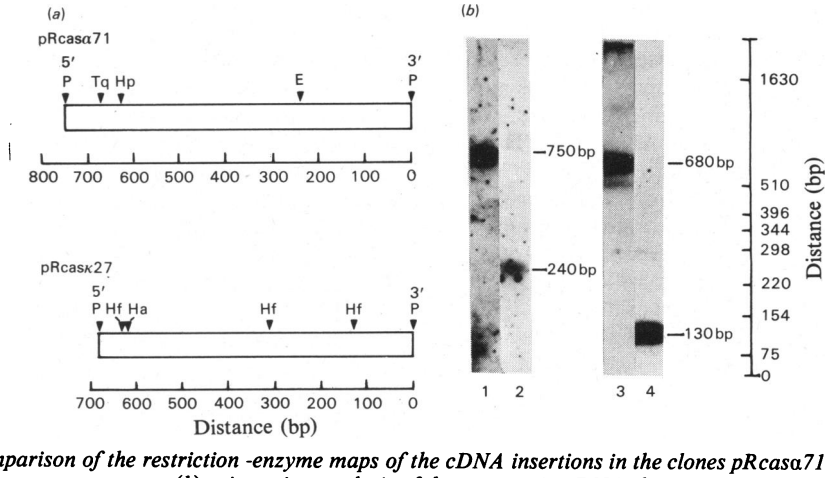

Fig. 6. (*a*) *Comparison of the restriction -enzyme maps of the cDNA insertions in the clones pRcasα71 and pRcasκ27 and* (*b*) *orientation analysis of the two casein-cDNA clones*
Abbreviations of the restriction enzymes are as follows: Hf, *Hinf*I; Hp, *Hpa*II; Tq, *Taq*I; Ha, *Hae*III; P, *Pst*I; E, *Eco*RII. (*b*) The gel-purified cDNA insertions of clone pRcasα71 (lanes 1 and 2) and clone pRcasκ27 (lanes 3 and 4) were electrophoresed undigested (lanes 1 and 3) or after digestion with *Eco*RII (lane 2) or *Hinf*I (lane 4). A Southern transfer was hybridized with end-labelled poly(U) to reveal the presence of 3′-terminal poly(A) sequences. The base-pair (bp) scale was derived from *Hinf*I digestion of plasmid pBR322.

rose electrophoresis into five major bands and several minor bands, a situation different from that reported for the guinea pig, in which three major mRNA bands were identified (Craig *et al.*, 1976). Translation *in vitro* of total cytoplasmic poly(A)$^+$ RNA revealed that more than 90% of the incorporated radioactivity was recovered in milk proteins irrespective of the amino acid precursor used. About 60% of this material was immunoprecipitated by the anti-(whole casein) serum. When [^{35}S]methionine-labelled translation products were immunoprecipitated with anti-(κ-casein) or anti-(x-casein) sera, a single band was observed on SDS/polyacrylamide-gel electrophoresis, whereas anti-(α-casein) serum yielded a doublet with M_r values of 29000 and 27000. Similar multiplicity of guinea-pig casein primary translation products has been described and characterized by using mRNA purified on immobilized casein-cDNA plasmids. The heterogeneity was rather specific to the reticulocyte-lysate cell-free system, which we used in this study, and was attributed to unscheduled modifications or signal-peptidase-like activity (Craig *et al.*, 1981; Pascall *et al.*, 1981). In the rabbit, heterogeneity was restricted to α-pre-casein translated in the absence of microsomal membranes, and therefore cannot be due to co- or post-translational modifications. It is unlikely that the two α-pre-casein bands are the product of two different mRNA species, because when products of translation *in vitro* of the same batch of poly(A)$^+$ RNA were precipitated under the same conditions, variable amounts were recovered in each band, suggesting variable extents of proteolytic degradation of the higher-M_r molecule. Such a proteolytic activity has been demonstrated in cell-free translation systems in the absence of microsomal membranes and shown to be inhibited by low concentrations of non-ionic detergents (Peeters *et al.*, 1979). Pre-α-lactalbumin represented the most abundant translated whey protein. Anti-transferrin serum, which reacted with serum transferrin (Dayal *et al.*, 1982), precipitated a band co-migrating with the secreted 80000-dalton milk iron-binding protein, indicating that transferrin is also a gene product of the mammary gland. Secretory component, a whey glycoprotein associated in milk with immunoglobulin A, was shown to be translated as larger precursor transmembrane proteins in a heterologous microsomal membrane system (Mostov *et al.*, 1980).

Comparison of the cell-free translation products and the corresponding secreted proteins (Table 1) did not provide definitive evidence as to the size of the pre-proteins, since the secretory products are variably glycosylated and phosphorylated (Dayal *et al.*, 1982). For α-lactalbumin, the M_r of the translated product (16000) is significantly smaller than that of the secreted molecule (22000), in contrast with that of the guinea pig (Craig *et al.*, 1976) and the human (Hall *et al.*, 1979). This discrepancy is explained by the high degree of glycosylation of rabbit α-lactalbumin (Hopp & Woods, 1979). For α-lactalbumin the size of the signal peptide was estimated to be 2000 daltons by comparing the M_r of the translated molecule (16000) with that of the sequenced protein (14052) (Hopp & Woods, 1979).

The casein-cDNA clones described in the present paper contain sequences representing portions of the α- and κ-casein-mRNA molecules respectively. Double-stranded cDNA made against total cytoplasmic polyadenylated RNA from rabbit lactating mammary gland revealed on neutral agarose gels a majority of molecules in the size range between approx. 600 and 900 base-pairs. Thus it seems that during cDNA synthesis casein-mRNA species were preferentially copied into molecules of two-thirds the full length. The presence in casein-mRNA molecules of regions containing rather stable secondary structures could be an explanation for this finding, as suggested by Hall *et al.* (1981) for human and for guinea-pig casein mRNA. The amino acid composition of rabbit caseins, which is reported in the accompanying paper (Dayal *et al.*, 1982), is consistent with this interpretation, since it indicates a high content of glutamic acid, proline, valine and leucine. Irrespective of codon usage, mRNA sequences encoding protein regions rich in the above amino acids are likely to form secondary structures stabilized by G–C pairings.

The casein-cDNA clones pRcasα71 and pRcasκ27 both contain 3′ sequences of the corresponding mRNA molecules. Comparison of their restriction-endonuclease cleavage sites therefore demonstrates that α- and κ-casein-mRNA molecules have different sequences. In addition, hybrid-promoted translation *in vitro* has also indicated the absence of extensive homology between sequences of α- and κ-casein-mRNA, since each cloned cDNA selected a distinct casein-mRNA species. Rabbit α- and κ-casein-mRNA molecules are of very similar size, although the length of their coding regions, as estimated from the molecular weight of their translation products *in vitro*, appears to differ by approx. 60 nucleotides.

Although we have not yet characterized β-casein-cDNA clones, our analysis of rabbit casein-mRNA species is consistent with the notion that three distinct casein proteins (Dayal *et al.*, 1982) are encoded by mRNA molecules that do not share regions of extensive homology. A similar situation has been described by Richards *et al.* (1981) for the three distinct casein-mRNA species of the rat lactating mammary gland.

We have also characterized a cDNA clone containing almost full-length sequences of the rabbit α-lactalbumin mRNA. Using this cDNA clone as a

probe, we have established that rabbit α-lactalbumin mRNA has a size of 550 nucleotides.

In conclusion, we have characterized the products of translation *in vitro* of the major rabbit milk-protein-mRNA species, including the three caseins and three whey proteins (α-lactalbumin, transferrin and secretory component). Two casein-cDNA and one α-lactalbumin-cDNA clones were characterized by hybrid-promoted translation, 'Northern' hybridization and restriction-enzyme mapping. One κ-casein-cDNA clone contained 60% of the corresponding mRNA sequence and one α-casein-cDNA clone was 65% of the α-casein-mRNA length. Two α-lactalbumin-cDNA clones were almost full length. The clones will serve as probes for further study of the hormonal regulation of these genes in primary cell cultures or in cell-free systems.

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