Studies on the Intracellular Segregation of Polyribosome-Associated Messenger Ribonucleic Acid Species in the Lactating Guinea-Pig Mammary Gland

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1. Free and membrane-bound polyribosomes were isolated and the associated mRNA species characterized by cell-free protein synthesis, RNA-complexity analysis and polyribosome run-off *in vitro*. 2. Of the recovered polyribosomal RNA 85% was associated with membrane-bound polyribosomes and contained 87-93% of the total milk-protein mRNA species as assessed by cell-free protein synthesis or RNA-complexity analysis. 3. RNA-complexity analysis showed that the abundant (milk-protein mRNA assumed) species constituted 55% of the post-nuclear poly(A)-containing RNA population, the remainder consisting of a moderately abundant population (18%) and a low-abundance population (27%). Calculations suggest that each population contained up to 2, 48 and 5000 different species respectively. 4. RNA-complexity analysis of the free polyribosomal poly(A)-containing RNA demonstrated that all the species in the post-nuclear fraction were present, though in different proportions, the abundant, moderately abundant and low-abundance groups representing 38, 30 and 32% of this population. 5. RNA-complexity analysis of the membrane-bound polyribosomal poly(A)-containing RNA revealed a more limited population, 72% consisting of the abundant (milk-protein mRNA) species, and 28% a population of up to 900 RNA species. 6. Polyribosome run-off confirmed that milk-protein mRNA was associated with the membrane-bound and free polyribosomes, but represented only a small fraction of the total protein synthesized by the latter. 7. Comparative analysis of milk proteins synthesized in mRNA-directed cell-free systems, or by run-off of free and of membrane-bound polyribosomes, is consistent with the interpretation that *in vivo* the initiation of protein synthesis occurs on free polyribosomes, followed by the attachment of a limited population to the endoplasmic reticulum. After attachment, but before completion of peptide synthesis, the detachable *N*-terminal peptide sequence of one of these (pre- α -lactalbumin) is removed. 8. The results are discussed in terms of the mechanisms involved in the intracellular segregation of mRNA species in the lactating guinea-pig mammary gland.

Protein synthesis in mammalian cells occurs on polyribosomes, some attached to membranes of the endoplasmic reticulum and some free in the cytoplasm. The proportion of polyribosomes associated with the endoplasmic reticulum varies widely in different cell types, but is particularly high in cells involved in the synthesis of secretory proteins (Palade, 1955).

In an extension to these observations it was proposed (Siekevitz & Palade, 1960) that the membrane-bound polyribosomes would be the site of synthesis of secretory proteins, whereas proteins required for intracellular functions would be synthesized on free polyribosomes. Biochemical studies have now

gone some way to substantiate this hypothesis and demonstrate that a functional specificity between the polyribosomal classes exists for variety of proteins, particularly in normal tissues. Studies have generally concerned the location of the site of synthesis of secretory proteins, though occasionally of non-secretory proteins. Thus in rat liver it has been shown that the great majority of the serum albumin is synthesized on membrane-associated polyribosomes, whereas ferritin is synthesized primarily on the free polyribosomes (see Redman, 1968; Hicks *et al.*, 1969). Studies on the location of the mRNA for the various proteins support these conclusions (Yap *et al.*, 1977; Zahring *et al.*, 1977).

Milk-protein synthesis is associated predominantly with the endoplasmic reticulum (see Denamur, 1974; Craig & Campbell, 1978). However, results concern-

Abbreviations used: cDNA, complementary DNA; SDS, sodium dodecyl sulphate.

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ing the precise proportion of the total poly(A)-containing milk-protein mRNA species associated with free or membrane-bound polyribosomes have been conflicting, in spite of the use of tissue from the normal lactating mammary gland. Moreover, as with other secretory protein systems, experiments have been designed to determine the relative intracellular distribution of only the 'abundant' mRNA species, which direct the synthesis of secretory proteins, but not the relative distribution of the less abundant mRNA species, which presumably direct the synthesis of the 'house-keeping' proteins, those proteins not secreted by the cell. Our own preliminary observations (Harrison *et al.*, 1976) demonstrated that, after subcellular fractionation of the lactating guinea-pig mammary gland, 80–85% of the recovered polyribosomal RNA was associated with the membrane-bound fraction, and the remainder with the free polyribosomal fraction. Poly(A)-containing RNA isolated from either fraction appeared to direct almost exclusively the synthesis of caseins in cell-free protein-synthesizing systems.

In this paper, using improved fractionation procedures, in combination with mRNA-directed cell-free protein synthesis, polyribosome run-off *in vitro* and RNA-complexity studies, we demonstrate that all poly(A)-containing RNA species isolated from the post-nuclear supernatant of the lactating guinea-pig mammary gland are represented in the free polyribosome fraction, whereas only a limited proportion of these are associated with membrane-bound polyribosomes. The latter, however, contain over 90% of the total abundant cellular polyribosome-associated poly(A)-containing RNA, which we assume directs the synthesis of the major guinea-pig milk proteins.

The results are discussed in terms of current concepts concerning the mechanisms involved in the intracellular compartmentalization of protein synthesis, and are consistent with the interpretation that, in the lactating guinea-pig mammary gland, (i) the initiation of protein synthesis occurs on free polyribosomes, (ii) although the majority of polyribosomes then attach to the endoplasmic reticulum, they represent only a limited proportion of the different poly(A)-containing RNA species within the mammary gland, though (iii) a very high percentage of the total poly(A)-containing RNA content.

Materials and Methods

Preparation of solutions and glassware

Preparation of buffers, scintillation fluid, all recrystallization and distillation procedures were as described by Craig *et al.* (1976), except that (i) buffers used during RNA-isolation procedures were autoclaved in the presence of 0.1% (v/v) diethyl pyro-

carbonate at 104 kPa (15 lb/in²) for 20 min, and (ii) all solutions required for nucleic acid hybridizations were passed through a small column of Chelex 100 and then autoclaved. Glassware used in cDNA preparations and nucleic acid hybridizations was silicone-treated, washed with Chelex-treated water and then sterilized overnight at 180°C before use.

Materials

Deoxyribonucleoside triphosphates, actinomycin D, d(pT)_{12–18} and proteinase K were purchased from Boehringer (London), Lewes, E. Sussex, U.K.; heparin (Li⁺ salt), calf thymus DNA type I, S₁ nuclease and aurointricarboxylic acid were from Sigma (London) Chemical Co., Fancy Road, Poole, Dorset BH17 7NH, U.K.; *NN'*-diallyltartardiamide was from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; sodium deoxycholate was from E. Merck Laboratory Chemicals, Darmstadt, W. Germany; and L-[4,5-³H]leucine (53 Ci/mmol), L-[³⁵S]methionine (700–1300 Ci/mmol) and deoxy[5-³H]cytidine 5'-triphosphate (22 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K.; avian myeloblastosis-virus reverse transcriptase (lot no. G-1976) was provided by Dr. J. W. Beard, Life Sciences Inc., St Petersburg, FL 33707, U.S.A.; all other chemicals and solvents were obtained from sources previously described (Craig *et al.*, 1976).

Buffers

Buffer A was 50 mM-Tris/HCl, pH 7.6 at 4°C, 0.15 M-sucrose, 25 mM-KCl, 5 mM-magnesium acetate.

Buffer B was buffer A containing 0.2 vol. of guinea-pig liver high-speed supernatant, and 250 µg of heparin/ml.

Buffer C was 50 mM-Tris/HCl, pH 7.6 at 4°C, 25 mM-KCl, 5 mM-magnesium acetate, 250 µg of heparin/ml.

Buffer D was buffer C containing 0.2 vol. of guinea-pig liver high-speed supernatant.

Buffer E was 20 mM-Tris/HCl, pH 7.5 at 20°C, 1 mM-EDTA.

Preparation of guinea-pig liver high-speed supernatant

Animals were killed by cervical dislocation, and the livers removed and washed in ice-cold buffer A. These were blotted, passed through a tissue mincer, and then disrupted in 2.5 vol. of buffer A by six strokes of a Teflon pestle (0.40 mm clearance) in a glass homogenizer tube operating at 1000 rev./min. The homogenate was then centrifuged at 12000g_{av.} for 10 min at 4°C, and the resulting post-mitochondrial supernatant centrifuged for a further 3 h at 120000g_{av.} at 4°C in the 60Ti rotor of a Beckman L5-65 ultracentrifuge. The upper two-thirds of the final supernatant was retained, stored at –70°C, and used as a source of liver ribonuclease inhibitor.

Preparation of low-salt free and membrane-bound polyribosomes from the post-nuclear supernatant of the lactating mammary gland

The isolation by differential centrifugation was similar to that described by Blobell & Potter (1967).

Glands from lactating guinea pigs 3–6 days *post partum*, killed by cervical dislocation, were dissected into ice-cold buffer B. The glands were rinsed in fresh buffer, and after removal of excessive fat and connective tissue were blotted, minced and then disrupted in 2.5 vol. of buffer B by two strokes of a Teflon pestle (0.40 mm clearance) in a glass homogenizer tube operating at 1000 rev./min. The homogenate was centrifuged at 800 g_{av} for 10 min at 4°C, and the resulting supernatant retained, filtered through glass wool to remove the upper lipid layer, and finally diluted to 20–30 A_{260} units/ml with buffer B.

Samples (18 ml) were then layered on to discontinuous sucrose gradients consisting of 6 ml of 1.0 M-sucrose in buffer B, layered over 12 ml of 2.3 M-sucrose in buffer B. The polyribosome fractions were then separated by centrifugation at 95 000 g_{av} for 18 h at 4°C in the SW27 rotor of a Beckman L2-65B ultracentrifuge. Membrane-bound polyribosomes were removed from the 1.0 M-/2.3 M-sucrose interface by using a Pasteur pipette, and either used immediately as a source of polyribosomal RNA, or, if required for sucrose-gradient or electron-microscopic analysis, were resuspended in buffer C, and finally collected by centrifugation at 95 000 g_{av} for 90 min at 4°C, in the 50 Ti rotor of a Beckman L5-65 ultracentrifuge.

To isolate the free polyribosomes, the remaining supernatant was carefully removed and discarded, and the walls of the centrifuge tubes were wiped clean. The sedimented free polyribosomes were then surface washed with several ml of buffer C and used immediately as a source of free polyribosomal RNA, or for sucrose-gradient or electron-microscopic analysis. For polyribosome run-off experiments, heparin was removed by resuspension of the polyribosomes in buffer C at 5–10 A_{260} units/ml, followed by centrifugation at 250 000 g_{av} for 3 h at 4°C in the SW56 rotor of a Beckman L5-65 ultracentrifuge through a 40% (w/v) sucrose cushion made up in 50 mM-Tris/HCl, pH 7.6 at 4°C, containing 25 mM-KCl and 5 mM-magnesium acetate. The sedimented polyribosomes were then resuspended by gentle hand homogenization at 13.4 A_{260} units/ml in 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH, pH 7.6, containing 120 mM-KCl, 5 mM-magnesium acetate and 6 mM-2-mercapto-ethanol, and added directly to the appropriate cell-free protein-synthesizing system.

Preparation of high-salt membrane-bound polyribosomes

Glands were minced and homogenized as described above in 2.0 vol. of buffer A made up to 250 mM-KCl

containing 0.2 vol. of liver supernatant and 250 μ g of heparin (Li^+ salt)/ml (breakage buffer). Nuclei and cell debris were then removed by centrifugation at 850 g_{av} for 10 min at 4°C, and a microsomal fraction was obtained by centrifuging the post-nuclear supernatant for a further 20 min at 20 000 g_{av} and 4°C. The sedimented high-salt microsomal fraction was then either used immediately as a source of membrane-bound polyribosomes in polyribosome run-off experiments, or alternatively further processed to remove free polyribosomes. This was achieved by gentle resuspension of the microsomal pellets with a vortex mixer in breakage buffer containing 2.1 M-sucrose (6 ml/g of starting tissue). Portions (10 ml) of the suspension were then transferred to Beckman SW27 tubes, and overlaid with 10 ml of breakage buffer containing 1.9 M-sucrose, and then 15 ml of breakage buffer containing 1.3 M-sucrose. This was then centrifuged to equilibrium at 100 000 g_{av} for 5 h at 4°C, resulting in the accumulation at the 1.3 M-/1.9 M-sucrose interface of the majority of the membranous material. This was removed with a Pasteur pipette, and used as a source of membrane-bound polyribosomal RNA.

For polyribosome run-off experiments, the microsomal fraction was resuspended by gentle hand homogenization in buffer C (1 ml/g of starting tissue), and membranous material solubilized by the addition of 0.2 vol. of 10% (v/v) Triton X-100 in buffer C. The polyribosomes were then centrifuged through a 40% (w/v) sucrose cushion in the absence of heparin as described above, and the sedimented polyribosomes resuspended at 13.4 A_{260} units/ml in 20 mM-Hepes/KOH, pH 7.6, containing 120 mM-KCl, 5 mM-magnesium acetate and 6 mM-2-mercapto-ethanol, and used immediately.

Sucrose-density-gradient analysis of polyribosomes

Sedimented free or membrane-bound polyribosomes were resuspended in buffer D at 40–50 A_{260} units/ml. The suspension was made 1.3% (w/v) with respect to sodium deoxycholate and 0.5 ml of the resulting suspension overlaid on 36 ml linear 10–40% (w/v) sucrose gradients in buffer C. The gradients were centrifuged for 2 h at 2°C and 95 000 g_{av} in the SW27 rotor of a Beckman L2-65B ultracentrifuge. Polyribosome profiles were monitored at 260 nm in the flow cell of a Unicam SP. 500 recording spectrophotometer. Gradients were pumped from the bottom of the tubes via a needle inserted through the gradients from above.

Isolation of poly(A)-containing RNA

Total post-nuclear poly(A)-containing RNA was isolated from the lactating guinea-pig mammary gland essentially as described previously (Craig *et al.*, 1976), except that heparin (100 μ g/ml) was included in the initial breakage buffer, and the post-nuclear

supernatant was incubated for 30 min at 45°C after the addition of NaCl and EDTA to 100 mM and 10 mM respectively, and SDS and proteinase K to final concentrations of 2% (w/v) and 250 µg/ml respectively. This was then extracted with phenol/chloroform (1:1, v/v) saturated with buffer E containing 100 mM-NaCl, and the RNA recovered by ethanol precipitation. Poly(A)-containing RNA was then isolated by affinity chromatography on oligo(dT)-cellulose as described previously (Craig *et al.*, 1976).

To obtain poly(A)-containing RNA from free or membrane-bound polyribosomes, each was resuspended at 10–20 A_{260} units/ml in buffer E containing 250 µg of proteinase K/ml. This was made 2% (w/v) with SDS and incubated for 30 min at 45°C before phenol/chloroform extraction; RNA was recovered by ethanol precipitation and the poly(A)-containing RNA isolated by chromatography on oligo(dT)-cellulose.

Rabbit globin mRNA was prepared from reticulocytes by affinity chromatography of polyribosomal RNA on oligo(dT)-cellulose as described by Aviv & Leder (1972), followed by sucrose-gradient centrifugation in the presence of formamide (see below). This was used for both cell-free protein synthesis and as a standard in RNA-complexity studies.

Electron-microscopic analysis of polyribosomes

Free and membrane-bound polyribosomal pellets were washed free of sucrose by resuspension in buffer C, and resedimented by centrifugation in the SW50 rotor of a Beckman L2-65B ultracentrifuge for 3 h at 4°C and 234 000 $g_{av.}$. The washed, sedimented polyribosomes were then fixed by using 2.0% (w/v) osmium tetroxide in 0.1 M-phosphate buffer, pH 7.4, dehydrated, embedded in Epon, sectioned, and finally stained with uranyl acetate and lead citrate before examination under the electron microscope.

Measurement of glucose 6-phosphatase activity

Glucose 6-phosphatase activity was determined by the measurement of the release of P_i . Assays containing 40 mM-glucose 6-phosphate, 7 mM-histidine monochloride, 1 mM-EDTA and 30 mM-sodium cacodylate, pH 6.5, were preincubated for 5 min at 37°C, and then incubated for a further 20 min at 37°C after the addition of the appropriate freshly prepared polyribosomal fraction. The reaction was terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid, denatured protein removed by centrifugation at 10 000 $g_{av.}$ for 10 min, and P_i then determined on 1.0 ml portions of the supernatant as described by Fiske & Subbarow (1925). One unit of glucose 6-phosphatase activity was expressed as the amount of enzyme that catalyses the hydrolysis of 1 µmol of substrate/20 min. Results are expressed as units per mg of protein.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as a standard.

Cell-free protein synthesis and product analysis

Wheat-germ S-30, Krebs II ascites-cell S-30 fraction and crude rabbit reticulocyte initiation factors were prepared as described previously (Craig *et al.*, 1976). mRNA-directed cell-free protein synthesis and antibody-precipitation procedures were also as described previously, and polyribosome run-off was performed under the ionic conditions required for mRNA-directed protein synthesis, but in the presence of aurintricarboxylic acid (30–50 µM) in an assay volume of 50 µl at 22°C for 30–45 min.

SDS/polyacrylamide-gel-electrophoretic analysis of [³⁵S]methionine-labelled cell-free products was performed in vertical (20.0 cm × 15.0 cm × 1.5 mm) slab gels by using a 1.5 cm 4% stacking gel and a 10% separating gel in a similar manner to that described previously (Craig *et al.*, 1976), but with *NN'*-diallyl-tartardiamide replacing *NN'*-methylenebisacrylamide on a molar basis. Electrophoresis at constant current was performed at 20 mA for 20 min and then at 40 mA for 18 h with circulating electrode buffer.

Gels were stained in Coomassie Brilliant Blue R, destained with 10% (v/v) acetic acid/10% (v/v) propan-2-ol, fluorographed by the procedure of Bonner & Laskey (1974), and exposed at –70°C with Fuji Rx medical X-ray film.

Size estimation of poly(A)-containing RNA

Polyacrylamide-gel electrophoresis in the presence of formamide was performed as previously described (Craig *et al.*, 1976).

Sucrose-gradient centrifugation in the presence of formamide was performed in 14 ml gradients consisting of 5–20% (w/v) sucrose gradients in 3 mM-Tris/HCl, pH 7.4 at 20°C, containing 3 mM-EDTA and 75% (v/v) formamide. Immediately before use the formamide was deionized by gentle stirring with Amberlite MB3 (10 g/100 ml) for 2 h at room temperature. RNA samples were freeze-dried, dissolved in 100–200 µl of 3 mM-Tris/HCl, pH 7.4, at 20°C containing 3 mM-EDTA and 75% (v/v) formamide, heated at 45°C for 15 min, and then applied to the top of the gradients. The samples were then centrifuged at 25°C and 182 000 $g_{av.}$ for 40 h, in the SW40 rotor of a Beckman L5-65 ultracentrifuge with the brake off. Gradients were fractionated by pumping out from the bottom of the tubes through a needle inserted from above. Fractions (0.5 ml) were monitored either at 280 nm in a Unicam SP.1800 recording spectrophotometer or alternatively assayed with [³H]poly(U) for the presence of poly(A)-containing RNA as described by Bishop *et al.* (1974a), after diluting each fraction with 0.03 M-sodium citrate/0.3 M-NaCl, so that the formamide was

present at a concentration of less than 15% (v/v) in the assay.

Synthesis of cDNA

High-specific-radioactivity ^3H -labelled DNA complementary to either total cytoplasmic, free or membrane-bound polyribosomal poly(A)-containing RNA was synthesized by our modification of the method described by Monahan *et al.* (1976a). Synthesis was carried out at 46°C for 20 min in volumes ranging from 50 μl to 1 ml and contained 50 mM-Tris/HCl, pH 8.3 at 46°C, 50 mM-KCl, 20 mM-dithiothreitol, 6 mM-magnesium acetate, 500 μM -dATP, 500 μM -dGTP, 500 μM -dTTP, 50 μM -dCTP, [^3H]dCTP (1 mCi/ml; 22 Ci/mmol), (dT)₁₂₋₁₈ (2.5 μg /ml), actinomycin D (50 μg /ml), poly(A)-containing RNA (20 μg /ml), avian-myeloblastosis-virus reverse transcriptase (200 units/ml) and 1.25% (v/v) ethanol. One unit of enzyme activity represents the incorporation of 1 mmol of dTMP into an acid-insoluble product in 10 min at 37°C.

The reaction was stopped by the addition of 0.1 vol. of 200 mM-EDTA and 0.05 vol. of 20% (w/v) SDS. Where necessary the reaction volumes were made up to 1.0 ml with 1.5 mM-sodium citrate containing 15 mM-NaCl, and then extracted with an equal volume of phenol/chloroform (1:1, v/v) saturated with 1.5 mM-sodium citrate containing 15 mM-NaCl. The aqueous and phenolic phases were separated by centrifugation at 800 g_{av} , at 10°C for 10 min, and upper aqueous phase was transferred to a silicone-treated polypropylene centrifuge tube suitable for use in a Beckman SW56 swing-out rotor. To this was added 0.1 vol. of 3 M-sodium acetate, pH 5.5, and 50 μg of *Escherichia coli* tRNA, and the nucleic acids were allowed to precipitate for 60 min at -70°C after the addition of 3 vol. of ethanol. The precipitated nucleic acid was then recovered by centrifugation at 120 000 g_{av} for 20 min at -5°C, the ethanol discarded and the sedimented nucleic acid dried briefly under vacuum. This was then dissolved in 0.5 ml of 0.1 M-NaOH containing 1 mM-EDTA, and the mixture incubated at 70°C for 20 min, then cooled to 20°C, and neutralized by the addition of 0.1 vol. of 1 M-sodium acetate, pH 4.5. Nucleotides were removed by gel filtration on a Sephadex G-50 column (0.8 cm $\times$ 17.0 cm) overlaying a 1 cm plug of Chelex. The column was pre-equilibrated and eluted at 60 ml/h in 10 mM-Tris/HCl, pH 7.4 at 20°C, containing 100 mM-NaCl, 1 mM-EDTA and 0.1% (w/v) SDS.

Fractions (0.5 ml) were collected, and those in the void volume containing trichloroacetic acid-precipitable ^3H -labelled material pooled, *E. coli* tRNA was added as co-precipitant (100 μg /ml of initial assay volume) and the nucleic acid precipitated overnight at -20°C in a silicone-treated Beckman SW40 tube after the addition of 0.1 vol. of 3 M-sodium

acetate, pH 5.5, and 3 vol. of ethanol. The precipitate was then recovered by centrifugation as described above, the supernatant discarded, and the precipitated nucleic acid dried under vacuum. This was then dissolved in 50–200 μl of sterile Chelex-treated double-distilled water, and stored at -70°C in 5–10 μl portions in sealed silicone-treated glass capillary tubes. Under these conditions 8–10% of the initial mRNA was transcribed into cDNA, of which 60–70% was recovered after alkaline hydrolysis and gel filtration.

Preparation of abundant cDNA species

^3H -labelled cDNA (8×10^5 c.p.m.; specific radioactivity 1.45×10^6 c.p.m./ μg) synthesized from total cytoplasmic poly(A)-containing RNA, and 1 μg of total cytoplasmic poly(A)-containing RNA, were freeze-dried separately in silicone-treated glass tubes. Each was dissolved in 500 μl of 10 mM-Hepes, pH 7.6, containing 0.6 M-NaCl and 1 mM-EDTA, and heated to 68°C. The contents were then mixed and hybridization was allowed to proceed to a R_{ot} value (Bishop *et al.*, 1974b) of 1.78 mmol of nucleotide $\cdot$ s $^{-1}$ $\cdot$ litre $^{-1}$ at 68°C. Ice-cold 100 mM-sodium acetate (1 ml), pH 4.6, containing 6 mM-ZnSO $_4$ was then added, and the mixture incubated for a further 30 min at 37°C in the presence of 100 units of S $_1$ nuclease. After the addition of 50 μg of *E. coli* tRNA, the mixture was then extracted once with phenol/chloroform as described previously, and the nucleic acid recovered by centrifugation at 120 000 g_{av} for 20 min at -5°C, after ethanol precipitation overnight at -20°C. This was then hydrolysed in 0.5 ml of 0.1 M-NaOH/1 mM-EDTA for 20 min at 70°C, neutralized by the addition of 0.05 ml of 1 M-sodium acetate, pH 4.5, and the abundant cDNA recovered by centrifugation after precipitation with 3 vol. of ethanol in the presence of 5 μg of *E. coli* tRNA as co-precipitant. This was then dissolved in 50 μl of Chelex-treated double-distilled water and stored in 5–10 μl batches in sealed capillaries at -70°C. Of the initial ^3H -labelled cDNA, 42–44% was resistant to S $_1$ -nuclease digestion after hybridization, and the recovery after alkaline hydrolysis and ethanol precipitation represented 75–80% of this material. One unit of S $_1$ nuclease will digest 1 μg of DNA to a HClO $_4$ -soluble form in 1 min at pH 4.6 at 37°C.

Hybridization of poly(A)-containing RNA with cDNA

Hybridizations were carried out in 0.24 M-sodium phosphate buffer, pH 6.8, containing 0.2% (w/v) SDS and 1 mM-EDTA over an RNA concentration range of 1 μg /ml to 1 mg/ml (1 A $_{260}$ unit $\equiv$ 40 μg of RNA) for periods of 10 s to 96 h. Routinely RNA sufficient for five to ten points was mixed with ^3H -labelled cDNA (2000 c.p.m. per point) and freeze-dried in a silicone-treated glass test tube. This was then redissolved in hybridization buffer (2.5 μl per

point) and 2.5 μ l samples were taken up and sealed in 5 μ l silicone-treated glass capillary tubes. These were then immersed in a water bath at 96°C for 5 min, and then rapidly transferred to an adjacent water bath at 70°C. After the appropriate hybridization period, tubes were removed, plunged into a salt/ice bath for 30–60s, and the contents blown into 1 ml of 100mM-sodium acetate, pH 4.6, containing 50mM-NaCl, 1mM-ZnSO₄, native calf thymus DNA (10 μ g/ml) and heat-denatured calf thymus DNA (10 μ g/ml). These were either quick-frozen in liquid nitrogen and stored at -20°C, or assayed immediately with S₁ nuclease to determine the extent of hybridization as follows.

Each tube was divided into two 450 μ l portions and 20 units of S₁ nuclease added to one of these. Both were then incubated at 37°C for 120 min. The incubations were terminated by the addition to each of 50 μ g of bovine serum albumin and 125 μ l of 50% (w/v) trichloroacetic acid, followed by 1 ml of 10% (w/v) trichloroacetic acid. After 30 min at 4°C, the resulting precipitates were collected on 2.5 cm Millipore GS filters (0.22 μ m) under vacuum, washed with 3 $\times$ 5 ml of 5% (w/v) trichloroacetic acid, dried for 60 min at 55°C, and then counted for radioactivity in 5 ml of toluene scintillation fluid. The percentage of S₁-nuclease-resistant [³H]cDNA radioactivity is equivalent to the percentage of [³H]cDNA hybridized.

Results

Isolation of free and membrane-bound polyribosomes

Our initial objective was to isolate two polyribosome populations, one containing only membrane-bound polyribosomes and the other containing only free polyribosomes.

In order to isolate undegraded polyribosomes from the post-nuclear supernatant of the lactating guinea-pig mammary gland, we have used a procedure based on that of Blobel & Potter (1967) involving sedimentation of the free polyribosomes through either a 2.0M- or 2.3M-sucrose cushion, in the presence of heparin, and a ribonuclease inhibitor present in a guinea-pig liver high-speed supernatant (Harrison *et al.*, 1976). Both ribonuclease inhibitors were required before intact polyribosomes containing undegraded mRNA could be isolated, particularly from the membrane-bound polyribosome fraction (see also Shafritz, 1974). Electron-microscopic examination of the 'free' polyribosomal fraction obtained by sedimentation through a 2.0M-sucrose cushion revealed not unexpectedly contamination of this fraction by mitochondria, though little evidence of membranous vesicle structures, typical of the endoplasmic reticulum. All such membrane and mitochondrial contaminants could, however, be removed by replacing the 2.0M-sucrose with a 2.3M-

sucrose cushion (see also Cioli & Lennox, 1973). Although this drastically decreased the apparent yield of free polyribosomes from 16% to 4% of the total recovered polyribosomal RNA, electron microscopy (Plate 1a) showed a free polyribosomal fraction devoid of mitochondrial and membranous vesicular structures, whereas the membrane-bound polyribosome fraction contained, as expected (Plate 1b), a predominance of membrane structures studded with clusters of ribosomes, and in addition deeply stained bodies, presumably casein micelles. Examination of each fraction for glucose 6-phosphatase activity, a marker enzyme for the endoplasmic reticulum (Nordlie, 1974), supported our electron-microscopic analysis, in that no activity could be detected in the free polyribosomal preparation, whereas significant activity of the enzyme could be

Fig. 1. Comparative size distribution of (a) free and (b) membrane-bound polyribosomes isolated from the lactating guinea-pig mammary gland

Polyribosomes from the post-nuclear supernatant fraction were isolated by discontinuous sucrose gradient centrifugation (1.0M-/2.3M-sucrose). The size distribution was determined by centrifugation on 10–40% (w/v) sucrose gradients in buffer C after pretreatment of the polyribosomes with sodium deoxycholate, precisely as described in the Materials and Methods section.

EXPLANATION OF PLATE I

Electron microscopy of free and membrane-bound polyribosome fractions isolated from the lactating guinea-pig mammary gland
Polyribosomes from the post-nuclear supernatant fraction were isolated by discontinuous sucrose-gradient centrifugation (1.0M-/2.3 M-sucrose) as described in the Materials and Methods section. These were then washed, fixed, embedded, sectioned and finally stained with uranyl acetate and lead citrate before examination under the electron microscope.
(a) Free polyribosomes; (b) membrane-bound polyribosomes.

detected in the membrane-bound polyribosomal fraction (5.6 units of P_i released/mg of protein).

Examination of the size distribution of both free and membrane-bound polyribosomes isolated in this manner by centrifugation on 10–40% sucrose gradients after prior treatment with sodium deoxycholate demonstrated the presence of large polyribosomal species, with a marked absence of monoribosomes and small polyribosomal species (Fig. 1). In the absence of deoxycholate, all membrane-bound polyribosomes sedimented to the bottom of the gradients, whereas the free polyribosomal profile remained unaltered.

As all isolation and analytical procedures were performed at low ionic strength (25 mM-KCl), so lessening the likelihood of contamination of the free polyribosome preparation with detached polyribosomes of membrane origin, it seemed reasonable to conclude that we had isolated a representative population of free polyribosomes. However, in order to obtain a representative fraction of membrane-bound polyribosomes, and also to quantify effectively the true ratio of membrane-bound to free polyribosomes in the isolated fractions, we have further modified our procedures. This has been necessary because of recent evidence, which has demonstrated, particularly in liver polyribosome preparations, that at low salt concentrations (25 mM-KCl) a large proportion of the free polyribosome population co-sediments with the membrane-bound polyribosomes (Ramsey & Steele, 1977). These represent a polyribosome population previously described as 'loosely' as opposed to 'tightly' attached membrane-bound polyribosomes (Rosbash & Penman, 1971).

However, on investigating the effect of increasing KCl concentration on the ratio of membrane-bound to free polyribosomes, when isolated from the post-nuclear supernatant of lactating guinea-pig mammary gland, by discontinuous sucrose-gradient centrifugation with a 2.0 M-sucrose cushion, we found that, unlike with rat liver, increasing KCl concentrations had little apparent effect on this ratio. Irrespective of the salt concentration, 14–16% of the recovered RNA was found associated with the free polyribosomes, and 84–86% with the membrane-bound polyribosomes. This confirmed previous estimates that in the lactating guinea-pig (Harrison *et al.*, 1976), ewe, rabbit and bovine mammary glands (see Denamur, 1974), in excess of 80% of the polyribosomal population was associated with the membrane-bound fraction. Consequently, in subsequent quantitative calculations of the proportion of different mRNA populations associated with each polyribosome fraction, we assume that 15.0% of the total are representative of the free polyribosomes, and 85.0% of the membrane-bound polyribosomes.

However, to ensure that our membrane-bound polyribosome preparation used for subsequent

analyses could not reasonably by current criteria be thought to contain significant amounts of contaminating free polyribosomes, all preparations of membrane-bound polyribosomes (see the Materials and Methods section) were isolated in the presence of high salt (205 mM-KCl), and the microsomal fraction was either used directly for polyribosome run-off experiments (after treatment with Triton X-100), or, if required for RNA-complexity and cell-free protein-synthesis studies, homogenized in 2.1 M-sucrose, and the membrane fraction separated from contaminating trapped free polyribosomes by discontinuous sucrose-gradient centrifugation in a similar manner to that described by Blobel & Dobberstein (1975a) and Mechler & Vassalli (1975).

Identification of poly(A)-containing mRNA species associated with membrane-bound and free polyribosomes by using cell-free protein synthesis

The ability of poly(A)-containing RNA species isolated from free and membrane-bound polyribosomes to direct cell-free protein synthesis was tested in both the wheat-germ and Krebs II ascites cell-free systems, under ionic conditions known to be optimal for milk-protein synthesis.

The addition of either of the poly(A)-containing RNA preparations to the wheat-germ cell-free system resulted in a 10–20-fold stimulation of the incorporation of [35 S]methionine into protein. Subsequent analysis of the protein by antibody precipitation, by using a mixture of antibodies raised against casein and α -lactalbumin, followed by SDS/polyacrylamide-slab-gel electrophoresis and fluorography, showed (Fig. 2) that whether the poly(A)-containing RNA was derived from a post-nuclear supernatant, free or membrane-bound polyribosomes four predominant protein species were synthesized and that all were precipitable with antibodies raised against milk proteins. These represent, as previously described (Craig *et al.*, 1976), the primary translation products of the three guinea-pig caseins (A, B and C) and of the whey protein, α -lactalbumin. Also as described previously, the caseins showed an anomalous mobility on SDS/polyacrylamide gels, in that the primary translation products (A', B', C') migrated faster than those of the mature secreted proteins, whereas α -lactalbumin was synthesized as pre- α -lactalbumin, and therefore migrated with a decreased mobility compared with the secreted protein.

A similar analysis of the proteins synthesized in the Krebs II ascites system directed by added poly(A)-containing RNA showed that, as with the wheat-germ cell-free system, independent of the source of lactating guinea-pig mammary-gland poly(A)-containing RNA, 85% or more of the total protein synthesized was precipitable by antibodies raised against casein or α -lactalbumin. In a parallel assay directed by a rabbit globin mRNA preparation, only

Fig. 2. SDS/polyacrylamide-gel analysis of [35 S]-methionine-labelled proteins synthesized in a wheat-germ cell-free protein-synthesizing system, directed by poly(A)-containing RNA isolated from the post-nuclear supernatant, free and membrane-bound polyribosomes of the lactating guinea-pig mammary gland

Cell-free protein synthesis, antibody precipitation, SDS/polyacrylamide-gel electrophoresis and fluorography were carried out as described in the Materials and Methods section. The relative electrophoretic mobilities of the authentic caseins (A, B etc.) and α -lactalbumin (α -LA) isolated from guinea-pig milk are indicated.

1.6% of the newly synthesized protein was so precipitated.

Although all poly(A)-containing RNA fractions directed the synthesis of milk proteins, it became apparent over a series of preparations that, if the same amount of poly(A)-containing RNA derived from either free or membrane-bound polyribosomes was added to the appropriate cell-free protein-synthesizing assay, that derived from the membrane-bound polyribosomes was always 2–3-fold more active than the equivalent preparation from free polyribosomes. Likewise, the poly(A)-containing RNA isolated from the post-nuclear supernatant was slightly less active than the equivalent membrane-bound polyribosome preparation, but markedly more active than the equivalent free polyribosome preparation. This was not apparently due to varying amounts of contaminat-

Fig. 3. Electrophoresis of lactating mammary-gland poly(A)-containing RNA species in the presence of formamide. Poly(A)-containing RNA isolated from the post-nuclear supernatant, free and membrane-bound polyribosomes was freeze-dried ($0.1 A_{260}$ unit each), dissolved in buffered formamide and electrophoresed on 4% polyacrylamide in the presence of formamide, and stained with Stainsall, as described in the Materials and Methods section. Rabbit reticulocyte 28S and 18S rRNA and *Escherichia coli* tRNA (4S) were electrophoresed ($0.1 A_{260}$ unit each) on parallel gels as marker species.

ing rRNA species, as comparative analyses of poly(A)-containing RNA species isolated from the post-nuclear supernatant, free or membrane-bound polyribosomes, by using polyacrylamide-gel electrophoresis in the presence of formamide, showed (Fig. 3) the absence from each fraction of detectable contaminating rRNA species, and the presence of three prominent RNA bands previously described (Craig *et al.*, 1976). Moreover, it was apparent, particularly in the free polyribosomal poly(A)-containing RNA preparation, that other RNA species were also present and that the milk-protein mRNA species were less predominant.

Quantification of the milk-protein mRNA activity associated with both polyribosome populations suggests that between 7% and 12% of the translatable milk-protein mRNA was associated with the free polyribosome fraction.

Analysis of the sequence complexity of poly(A)-containing RNA species isolated from the post-nuclear supernatant, free and membrane-bound polyribosomes of the lactating guinea-pig mammary gland

The presence of poly(A)-containing RNA species directing the synthesis of milk proteins could be demonstrated by the use of cell-free systems, but very few other proteins appeared to be synthesized. Consequently we have investigated the sequence complexity of the poly(A)-containing RNA population isolated from the post-nuclear supernatant, the free (low-salt) and the membrane-bound (high-salt) polyribosomes, of the lactating guinea-pig mammary gland, by using the RNA excess hybridization technique first described by Bishop *et al.* (1974b).

cDNA copies of each poly(A)-containing RNA population were prepared by using avian-myeloblastosis-virus reverse transcriptase and [^3H]dCTP as the radiolabelled deoxyribonucleoside triphosphate. The cDNA had a final specific radioactivity of 3.37×10^7 d.p.m./ μg , and ranged in size from 400 to 1300 nucleotides as judged by alkaline-agarose-gel electrophoresis (McDonnell *et al.*, 1977) by using ^{32}P -labelled *Hpa*II-restricted polyoma DNA fragments as molecular-weight markers (L. Hall, D. Parker & R. Craig, unpublished work); 4–7% remained resistant to S_1 nuclease after incubation in the absence of the homologous RNA templates. High-specific-radioactivity [^3H]cDNA was also prepared from highly purified rabbit globin mRNA. This was essentially full length (650 nucleotides) when analysed by alkaline agarose-gel electrophoresis as described above.

Using these high-specific-radioactivity [^3H]cDNA hybridization probes, we have compared the kinetics of hybridization of poly(A)-containing RNA isolated from the three RNA populations, each against its homologous cDNA. To obtain relative complexity values for the different abundance classes, and so to allow comparative analysis of the resulting data with other published results, we have compared the relative rates of hybridization (R_{ot}) of each class with that obtained for an RNA population of known complexity, in this case rabbit globin mRNA. Globin mRNA consists of a mixture of α - and β -globin mRNA species with a total complexity of about 1300 nucleotides. In our hands this had a R_{ot} of $0.815 \text{ mmol of nucleotide} \cdot \text{litre}^{-1} \cdot \text{s}^{-1}$ (Fig. 4). Consequently the relative complexity of any single RNA or mixture of RNA species may be determined by comparing the R_{ot} of the unknown with that of the globin standard. Furthermore, provided that the average molecular weight of an RNA population is also known, it is possible to estimate with a reasonable degree of accuracy the number of RNA sequences present in a given population (see Bishop *et al.*, 1974b).

As expected, hybridization of cDNA, prepared

Fig. 4. RNA-complexity analysis of poly(A)-containing RNA species isolated from the post-nuclear supernatant of the lactating mammary gland

Poly(A)-containing RNA from the post-nuclear supernatant was hybridized to (a) its homologous cDNA at RNA concentrations of $1 \mu\text{g/ml}$ (■) and $50 \mu\text{g/ml}$ (□) respectively, and (b) the abundant cDNA at $2.5 \mu\text{g/ml}$ (▲). The hybridization curve of rabbit globin mRNA, $0.2 \mu\text{g/ml}$ (●) and $1 \mu\text{g/ml}$ (○), against its homologous cDNA was included as a kinetic standard. Line fitting of these and all subsequent curves was by computer. R_{ot} 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.

from poly(A)-containing RNA isolated from the post-nuclear supernatant, against an excess of its homologous RNA was markedly slower than that of an equivalent globin-mRNA hybridization analysis. Moreover, unlike the globin mRNA, which hybridized over nearly two orders of magnitude of R_{ot} , the post-nuclear poly(A)-containing RNA hybridized with three distinct transitions (Fig. 4) over approximately five orders of magnitude of R_{ot} , indicating that there existed three distinct abundance classes of poly(A)-containing RNA. In order to determine the true R_{ot} value for each class (and subsequently both the relative complexity and the approximate number of RNA species present in each) we have applied a computer program for least-squares analysis of reassociation and hybridization data (Pearson *et al.*, 1977; Higgins *et al.*, 1978). This not only provides the best fit for the appropriate number of components, but also adjusts the observed R_{ot} values to allow for the fact that each class of RNA represents only a fraction of the total RNA present. From the corrected R_{ot} values, the total complexity (in nucleotides) of each abundance class may then be calculated by using globin mRNA as a standard. Provided that the number-average molecular weight of the population has been previously determined, the number of species present may also be calculated with reasonable accuracy.

Fig. 5. Estimation of the number-average molecular weight of the poly(A)-containing RNA species present in the post-nuclear supernatant of the lactating mammary gland

The size distribution of poly(A)-containing RNA from the post-nuclear supernatant was determined by centrifugation on 5–20% (w/v) sucrose gradients in the presence of 75% formamide (see the Materials and Methods section). The distribution of the poly(A)-containing RNA species was determined by hybridization to $[^3\text{H}]\text{poly}(\text{U})$ ($\square$), and average sedimentation coefficient determined from a cumulative plot (inset) of the $[^3\text{H}]\text{poly}(\text{U})$ hybridized (c.p.m.), by using rabbit reticulocyte 28S and 18S rRNA and *E. coli* tRNA (4S) centrifuged on parallel gradients as marker species. For further comparison rabbit globin mRNA (10S) was also analysed and its position determined by hybridization to $[^3\text{H}]\text{poly}(\text{U})$ ($\blacksquare$). The number-average molecular weight was then determined from the sedimentation coefficient by using the formulae of Spirin (1963).

To determine the number-average molecular weight we have applied the formulae of Spirin (1963), after first determining the size distribution of the post-nuclear poly(A)-containing RNA population across a formamide/sucrose gradient (Fig. 5) by using $[^3\text{H}]\text{poly}(\text{U})$ as a hybridization probe, and 28S and 18S rabbit reticulocyte rRNA, and rabbit globin mRNA, centrifuged in parallel as marker species. If we assume that no extensive size variation in the poly(A) tract size occurs across the gradient, then the number-average molecular weight may be calculated from a cumulative plot of the fraction of poly(A)-containing RNA molecules against their nucleotide chain length (as determined from the sedimentation coefficient). In this manner, an average size of 1350 nucleotides (14.6S) was determined for the post-nuclear poly(A)-containing RNA species of the lactating guinea-pig mammary gland. This value was also used to approximate the number of species

present in the poly(A)-containing RNA isolated from the free and the membrane-bound polyribosome species.

Using this value, we could determine (Table 1) that the most abundant class, representing 55% of the total hybridized population, consisted of approximately one or two RNA species, whereas the intermediate or moderately abundant class, representing 18% of the population, was composed of 16–48 RNA species, and the least abundant class consisted of 1816–4668 RNA species and represented 27% of the population. Complexity analysis of the free polyribosomal poly(A)-containing RNA in the identical manner again revealed three complexity classes of RNA, each of similar complexity to the total cytoplasmic poly(A)-containing RNA, but present in different relative proportions (Fig. 6a, Table 1), the abundant, moderately abundant and least abundant classes being represented by approx.

Table 1. Numerical analysis of the hybridization kinetics of poly(A)-containing RNA isolated from the post-nuclear supernatant, membrane-bound and free polyribosomes of the lactating mammary gland, back-hybridized to their homologous cDNA preparations

Hybridization procedures were as described in the Materials and Methods section. The RNA complexity (nucleotides) of each abundance group has been determined relative to a rabbit globin mRNA standard (1300 nucleotides assumed). All calculations are based on $R_{ot\frac{1}{2}}$ values (Bishop *et al.*, 1974b) obtained after correction for the fraction of RNA represented by that class. The number of sequences present in each abundance class was determined by dividing the resulting complexity values (in nucleotides) by the number-average molecular weight. Results are expressed as means $\pm$ s.d. determined by computer (see Higgins *et al.*, 1978).

Source of poly(A)-containing RNA	Transition	$R_{ot\frac{1}{2}}$	$R_{ot\frac{1}{2}}$ corrected	Complexity (nucleotides)	Estimated number of species	Relative % of total poly(A)-containing RNA
Rabbit globin mRNA	1	8.4×10^{-4}	$8.15 \times 10^{-4} \pm 5.81 \times 10^{-5}$	1300	—	100
Total post-nuclear supernatant	1	3.98×10^{-3}	$1.31 \times 10^{-3} \pm 2.51 \times 10^{-4}$	2092	1.54 ± 0.3	55
	2	4.22×10^{-1}	$2.69 \times 10^{-2} \pm 1.10 \times 10^{-2}$	42940	32 ± 16	18
	3	10.6	2.74 ± 1.06	4378000	3242 ± 1426	27
Free polyribosomes	1	5.01×10^{-3}	$7.66 \times 10^{-4} \pm 1.78 \times 10^{-4}$	1222	0.91 ± 0.21	38
	2	4.22×10^{-1}	$6.47 \times 10^{-2} \pm 2.08 \times 10^{-2}$	103200	76 ± 24	30
	3	10	$2.11 \pm 4.24 \times 10^{-1}$	3360000	2488 ± 497	32
Membrane-bound polyribosomes	1	3.55×10^{-3}	$1.63 \times 10^{-3} \pm 2.19 \times 10^{-4}$	2603	1.93 ± 0.25	72
	2	2.51	$5.87 \times 10^{-1} \pm 1.93 \times 10^{-1}$	927100	694 ± 231	28

Fig. 6. RNA-complexity analysis of poly(A)-containing RNA species isolated from free and membrane-bound polyribosomes of the lactating mammary gland (a) Poly(A)-containing RNA from free polyribosomes was hybridized to (i) its homologous cDNA at RNA

1, 52–100 and 1991–2985 species respectively, with each class contributing 38, 30 and 32% of the total population. However, complexity analysis of the membrane-bound-polyribosome poly(A)-containing RNA revealed two classes only (Fig. 6b, Table 1), the most abundant contributing 72% of the total hybridized population and representing approximately two species, whereas the remaining 28% was represented by a class of 463–925 RNA species.

In order to assess whether or not the abundant RNA species present in the post-nuclear, the membrane-bound and free polyribosome fractions were composed of the same RNA sequences, the abundant cDNA fraction present in the total post-nuclear cytoplasmic fraction was isolated (see the Materials and Methods section) and then back-hybridized to all three poly(A)-containing RNA fractions (Figs. 4, 6a, 6b). In each case the hybridization went to completion with a single inflexion at the expected $R_{ot\frac{1}{2}}$ value, confirming that RNA species present in the free and membrane-bound polyribosome poly(A)-containing RNA contained the same abundant poly(A)-containing RNA sequences as found in the total post-nuclear preparation, a result consistent

concentrations of 1 µg/ml (■), 10 µg/ml (▽) and 50 µg/ml (▲), and (ii) the abundant cDNA at 5 µg/ml (●). (b) Poly(A)-containing RNA from membrane-bound polyribosomes was hybridized to (i) its homologous cDNA at RNA concentrations of 1 µg/ml (●), 10 µg/ml (○) and 50 µg/ml (□), and (ii) the abundant cDNA at 1 µg/ml (△) and 5 µg/ml (▲).

with the presence of milk-protein mRNA species (the abundant mRNA) on both membrane-bound and free polyribosomes. Quantification of these data suggests that in the lactating gland 8–10% of the total cytoplasmic milk-protein mRNA was associated with the free polyribosomes.

In addition to confirming that the milk-protein-mRNA species were associated with both polyribosomal fractions, though in markedly differing amounts, RNA-complexity studies demonstrate that the number of poly(A)-containing RNA species associated with the membrane-bound polyribosomes represents a relatively small proportion of the total cytoplasmic sequences, perhaps 20%, whereas all post-nuclear poly(A)-containing RNA species appear to be represented on free polyribosomes.

We have performed also a series of control back-hybridization experiments to determine whether (i) all poly(A)-containing RNA sequences present on

membrane-bound polyribosomes were present on free polyribosomes, and (ii) whether the poly(A)-containing RNA sequences found on free polyribosomes were representative of the total cytoplasmic poly(A)-containing RNA population. The results (Fig. 7) show that all poly(A)-containing RNA species derived from membrane-bound polyribosomes are present in significant quantities in free polyribosomes, and that all the sequences present in a post-nuclear poly(A)-containing RNA preparation are also associated with free polyribosomes. In each experiment hybridization went to completion, though the kinetics differed slightly from those experiments using homologous RNA. As a final control, back hybridization of poly(A)-containing RNA isolated from membrane-bound polyribosomes against [3 H]-cDNA synthesized from free polyribosomal poly(A)-containing RNA confirmed (i) the limited nature of sequences associated with the endoplasmic reticulum, the hybridization not going to completion, and (ii) the presence of only two abundance classes in this population (Fig. 8).

Analysis of protein species synthesized in vivo by free and membrane-bound polyribosomes, using polyribosome run-off in vitro

The following experiments were designed (i) to determine whether the numerous poly(A)-containing RNA species detected by RNA-complexity studies represent active mRNA molecules which direct the synthesis of proteins other than milk proteins, or (ii) to consider the possibility that the milk-protein

Fig. 7. Hybridization of cDNA prepared from poly(A)-containing RNA from the post-nuclear supernatant and membrane-bound polyribosomes of the lactating gland with heterologous poly(A)-containing RNA isolated from free polyribosomes

Free polyribosomal poly(A)-containing RNA was hybridized to cDNA synthesized from poly(A)-containing RNA from (a) the post-nuclear supernatant or (b) membrane-bound polyribosomes at RNA concentrations of 1 μ g/ml (■), 10 μ g/ml (□) and 50 μ g/ml (●).

Fig. 8. Hybridization of cDNA prepared from free polyribosomal poly(A)-containing RNA with poly(A)-containing RNA isolated from membrane-bound polyribosomes

Membrane-bound polyribosomal poly(A)-containing RNA was hybridized to cDNA synthesized from free polyribosomal poly(A)-containing RNA, at RNA concentrations of 1 μ g/ml (■), 10 μ g/ml (□) and 50 μ g/ml (●).

mRNA species identified in the free polyribosomal fraction may not be associated with polyribosomes, but represent mRNA species in the form of messenger ribonucleoprotein particles that have co-purified during the isolation procedures.

In order to achieve these objectives we have isolated Triton X-100-treated (high-salt) membrane-bound polyribosomes and free (low-salt) polyribosomes. These we have run off in a wheat-germ cell-free protein-synthesizing system, in both the presence and the absence of aurintricarboxylic acid, an inhibitor of the initiation of protein synthesis (Lodish *et al.*, 1971), under ionic conditions identical with those required for mRNA-directed milk-protein synthesis. As a control to ensure that no initiation *de novo* of mRNA not previously associated with polyribosomes was occurring, total post-nuclear poly(A)-containing RNA isolated from the lactating mammary gland was assayed in parallel, in the presence and absence of the same inhibitor. In all experiments, the concentration of aurintricarboxylic acid required to inhibit initiation was kept to a minimum (30–50 μM), the final concentration depending on the amount of wheat-germ S-30 fraction present in the assays.

The effect of aurintricarboxylic acid on the

initiation of mRNA-directed protein synthesis and its effect or otherwise on the run-off of membrane-bound and free polyribosomes were examined initially over a time course (Fig. 9). At concentrations of inhibitor that completely prevented mRNA-directed protein synthesis, only a limited inhibition of the incorporation of [^3H]leucine into protein was observed during run-off of the Triton X-100-treated membrane-bound polyribosomes. However, in contrast, the total amount of protein synthesized by the free polyribosomal fraction under similar conditions was decreased by as much as 30%. This suggests that in the free-polyribosome fraction some mRNA activity present in the assay may not be associated with guinea-pig ribosomes, and consequently may contribute to the large amount of milk-protein mRNA detected in the free polyribosomal preparation, as assessed by using mRNA-directed cell-free protein-synthesizing systems and RNA-complexity studies.

Analysis of the [^{35}S]methionine-labelled polyribosome run-off products by SDS/polyacrylamide-slab-gel electrophoresis followed by fluorography confirmed that the free polyribosomes (Fig. 10) were the site of synthesis of a very great variety of peptides (mol.wt. 12000–150000), and also demonstrated that proteins with the expected electrophoretic mobility

Fig. 9. Time course of polyribosome run-off in the wheat-germ cell-free protein-synthesizing system in the presence and absence of aurintricarboxylic acid

The incorporation of [^3H]leucine into protein was determined over a time course in the presence and absence of aurintricarboxylic acid (30 μM), by using a wheat-germ cell-free system directed by (a) endogenous activity, (b) poly(A)-containing RNA from the post-nuclear supernatant of the lactating mammary gland, (c) lactating mammary-gland free polyribosomes, (d) lactating mammary-gland membrane-bound polyribosomes. $\blacktriangle$, 30 μM -aurintricarboxylic acid; $\square$, no aurintricarboxylic acid.

Fig. 10. SDS/polyacrylamide-gel analysis of [^{35}S]methionine-labelled proteins synthesized in the wheat-germ cell-free system after run-off of lactating mammary-gland free polyribosomes

Free polyribosomes (low salt) and poly(A)-containing RNA from the post-nuclear supernatant of the lactating mammary gland were both incubated in the presence and absence of $30\text{ }\mu\text{M}$ -aurintricarboxylic acid (ATA) in a wheat-germ cell-free system for 45 min at 22°C . Products were separated and the milk proteins identified as described in Fig. 2.

and distribution of the primary milk-protein translation products were synthesized in both the presence and the absence of aurintricarboxylic acid in this preparation. These were precipitable with a mixture of antisera that had been raised against guinea-pig caseins A, B, C and α -lactalbumin, thus providing direct evidence that milk-protein mRNA species are present on free polyribosomes.

Similar analysis of the membrane-bound-polyribosome run-off products (Fig. 11) revealed a less complex pattern of peptides, the predominant ones being the milk proteins, with only a very small proportion of peptides of higher molecular weight. However, in addition to these quantitative differences, the apparent distribution of the caseins after run-off of membrane-bound polyribosomes was markedly different from that obtained either after mRNA-directed protein synthesis in the wheat-germ cell-free system, or by run-off of free polyribosomes, casein

B being particularly prominent, and casein C markedly less intense. Moreover, although pre- α -lactalbumin was clearly present, an additional antibody-precipitable band was also apparent which co-electrophoresed with α -lactalbumin, thus providing evidence that the 'signal' peptide of pre- α -lactalbumin is removed during on-going translation on membrane-bound polyribosomes *in vivo*. Similar evidence of processing of the caseins was not apparent, though the possibility cannot be discounted. All primary translational products of guinea-pig caseins are synthesized with *N*-terminal extensions, and the processing of these is a membrane-requiring event (Craig *et al.*, 1979b). Unfortunately, although the polyacrylamide-gel system that we have chosen (Weber *et al.*, 1972) to separate the peptides in this series of experiments clearly resolves both the mature milk proteins and the primary translation products synthesized *in vitro*, the resolution has not proved

Fig. 11. SDS/polyacrylamide-gel analysis of [³⁵S]methionine-labelled proteins synthesized in the wheat-germ cell-free system after run-off of lactating mammary-gland membrane-bound polyribosomes. Membrane-bound polyribosomes (high salt) and poly(A)-containing RNA from the post-nuclear supernatant of the lactating mammary gland were both incubated in the presence and absence of 30 μ M-aurintricarboxylic acid (ATA) in a wheat-germ cell-free system for 45 min at 22°C. Products were separated and the milk proteins identified as described in Fig. 2.

sufficient to resolve, with the exception of pre- α -lactalbumin and α -lactalbumin, small differences in apparent molecular weight of the caseins caused by processing or other post-translational modification events. Other polyacrylamide-gel systems give better resolution of the peptides synthesized *in vitro* (see Zehavi-Willner & Lane, 1977), but do not resolve two of the secreted peptides, caseins A and B (Craig *et al.*, 1978). Further evidence for the presence of milk-protein mRNA species on free polyribosomes was obtained by hybridization of free polyribosomal RNA to the abundant cDNA species. In this experiment free (low-salt) polyribosomes were sized across a sucrose gradient without prior treatment with detergent, fractionated, and the polyribosomal RNA was recovered from each fraction. R_{0t} curves were then constructed for each fraction by using the abundant [³H]cDNA as the hybridization probe. The result (Fig. 12) confirmed that these sequences could be

identified throughout the gradient, though they were present in their greatest relative concentration in fractions 2, 3 and 4 (R_{0t} of 0.98, 1.10 and 1.12 mol of nucleotide $\cdot$ litre⁻¹ $\cdot$ s⁻¹ respectively) and at lower concentration at the top and bottom of the gradient (R_{0t} of 1.54 and 1.80 mol of nucleotide litre⁻¹ $\cdot$ s⁻¹ respectively), results consistent with the presence of milk-protein mRNA species predominantly on the smaller free polyribosomes, though in significant amounts on larger polyribosomes.

Discussion

Our previous work on the isolation and characterization of milk-protein mRNA from the lactating mammary gland of the guinea pig demonstrated that in the wheat-germ cell-free protein-synthesizing system the whey protein α -lactalbumin was synthesized as a precursor polypeptide extended at the *N*-terminus (pre- α -lactalbumin). The position with respect to the three caseins was less clear, owing to

Fig. 12. Distribution of milk-protein mRNA sequences on free polyribosomes

Free (low-salt) polyribosomes were prepared and their size distributions determined across a 10–40% (w/v) sucrose gradient in buffer C as described in Fig. 1, but without prior treatment with sodium deoxycholate. The gradient fractions were divided into five equal parts, pooled, and the RNA was extracted with phenol/chloroform after digestion with proteinase K. The relative amounts of milk-protein mRNA sequences in the total polyribosomal RNA was determined by hybridization to the abundant cDNA, as described in the Materials and Methods section, at RNA concentrations of 50 µg/ml (■) and 500 µg/ml (●).

their anomalous mobilities on SDS/polyacrylamide-gel electrophoresis compared with the caseins isolated from guinea-pig milk.

It is now known that, in the absence of membrane fragments, in addition to guinea-pig, rat and sheep α -lactalbumins (Craig *et al.*, 1976; Lingappa *et al.*, 1978; Mercier *et al.*, 1978), guinea-pig and sheep caseins are also synthesized *in vitro* as precursor proteins with *N*-terminal extensions (Gaye *et al.*, 1977; Mercier *et al.*, 1978; Craig *et al.*, 1979*b*). Moreover, the anomalous electrophoretic mobility of the caseins synthesized *in vitro* when separated by SDS/polyacrylamide-gel electrophoresis reflects the absence from established cell-free protein-synthesizing systems of certain post-translational modification events, in particular phosphorylation (A. P. Boulton, J. C. Pascall, C. D. Lane & R. K. Craig, unpublished work).

The identification of pre-proteins is consistent with results described for many other secretory systems (Devillers-Thiery *et al.*, 1975; Chan *et al.*, 1976; Kemper *et al.*, 1976; Maurer *et al.*, 1977; Strauss *et al.*, 1977), which demonstrate that, with the major exception of ovalbumin (Palmiter *et al.*, 1978), secretory proteins are synthesized with a hydrophobic signal sequence 15–30 amino acids in length, results consistent in principle with the signal hypothesis (Blobel & Sabatini, 1971; Milstein *et al.*, 1972; Blobel & Dobberstein, 1975*a,b*). A prediction of this hypothesis is that the initial step involved in the intracellular segregation of proteins destined for intra- or extra-cellular functions requires that initiation of protein synthesis should occur on free polyribosomes. Subsequently, after the synthesis of the initial 40–50 amino acid residues, those nascent polypeptide chains that contain a hydrophobic signal

sequence bind to membrane receptor sites on the endoplasmic reticulum. Continued protein synthesis results in vectorial transport across the membrane followed by proteolytic cleavage of the 'signal' sequence, either during or after the completion of peptide synthesis.

Although it has proved possible to demonstrate *in vitro*, by using synchronized mRNA-directed cell-free systems in the presence and absence of membrane fragments, that membrane-bound polyribosomes engaged in the synthesis of secretory and integral viral membrane proteins are derived from free polyribosomes (Rothman & Lodish, 1977; Boime *et al.*, 1977), evidence that might suggest that this occurs *in vivo* is less definitive, although such a mechanism was first proposed by Rosbash (1972).

Preliminary work from our laboratory (Harrison *et al.*, 1976) demonstrated that mRNA species isolated from free and membrane-bound polyribosomal fractions of the lactating guinea pig directed the synthesis of caseins. We have now shown that the addition of poly(A)-containing RNA species isolated from both the free and membrane-bound polyribosomes to mRNA-directed cell-free protein-synthesizing systems results in the almost exclusive synthesis of milk proteins, with little evidence for the presence of other poly(A)-containing mRNA species, presumably required to direct the synthesis of the 'house-keeping' proteins, particularly on the free polyribosomes. However, the presence of other mRNA and poly(A)-containing RNA species in either polyribosomal fraction could be demonstrated by using either RNA-complexity studies or polyribosomes run off *in vitro*. These observations substantiate and extend the work of Tse & Taylor (1977), who showed in experiments concerning the quantification of albumin mRNA that factors other than specific mRNA contents influence quantification experiments dependent on the synthesis of specific proteins in cell-free systems.

Results from RNA-complexity studies confirm that the lactating guinea-pig mammary-gland post-nuclear poly(A)-containing RNA population, in common with that from many other cell types and tissues studied (see Bishop *et al.*, 1974b; Campo & Bishop, 1974; Ryffel & McCarthy, 1975; Williams & Penman, 1975; Axel *et al.*, 1976; Hastie & Bishop, 1976; Monahan *et al.*, 1976b; Young *et al.*, 1976; Sippel *et al.*, 1977; Parker & Mainwaring, 1977; Higgins *et al.*, 1978), contained three abundance groups, and that, as with other hormonally regulated tissues, such as the chick oviduct (Monahan *et al.*, 1976b) and rat prostate (Parker & Mainwaring, 1977), a very high proportion of the total poly(A)-containing RNA population from the lactating mammary gland consisted of only a few sequences. These, we assume, correspond to the milk-protein mRNA species. Assessment of the number of sequences present in each abundance group can only

at best be approximate, particularly for the low-abundance sequences, where a small variation in the R_{ot_1} results in large differences in the estimated number of RNA sequences present. Similarly, differing extents of rRNA contamination from preparation to preparation (we have made no allowance for this, as estimations suggest less than 10% rRNA) will affect the absolute numbers of sequences in each class, as will assumptions about the efficiency of cDNA synthesis for different poly(A)-containing RNA populations by the reverse transcriptase reaction, and the size of the resulting cDNA transcripts (Hastie & Bishop, 1976).

However, allowing for the possible variations caused by correction factors and assumptions, and bearing in mind the disparity in numbers reported by different laboratories for the same cell line (see Bishop *et al.*, 1974b; Williams & Penman, 1975), the results that we have presented are consistent with the presence of up to 5000 poly(A)-containing RNA species in the post-nuclear supernatant of the lactating guinea-pig mammary gland, distributed between three abundance groups, of which the most abundant represents as much as 55% of the total population. These values are similar to those reported by Parker & Mainwaring (1977) for the complexity of total cellular poly(A)-containing RNA isolated from the rat ventral prostate.

The extension of this technique to compare and contrast the poly(A)-containing RNA species present in the free and membrane-bound polyribosome populations, both with each other and with the post-nuclear fraction, confirmed the presence of the abundant poly(A)-containing fraction in both the free and the membrane-bound polyribosomes. These experiments also demonstrate that, although all the poly(A)-containing RNA sequences present in the membrane-bound polyribosomes are also present associated with the free polyribosomes, these represented only 20% of the sequences present in post-nuclear supernatant, though 80–85% of the total polyribosomal poly(A)-containing RNA.

These results demonstrate that in the lactating guinea-pig mammary gland a clear-cut functional distribution occurs between the polyribosome classes, and support the concepts that:

(i) the initiation of all protein synthesis occurs on free polyribosomes;

and (ii) only a limited population of these polyribosomes subsequently attach to the putative membrane receptor sites of the endoplasmic reticulum.

Furthermore, the results demonstrate that, in addition to the abundant secretory protein mRNA species, found predominantly associated with the endoplasmic reticulum, a second relatively low-abundance group is also associated with the membrane-bound polyribosomes. What these represent must remain

conjecture, though we speculate that they may well direct the synthesis of integral membrane proteins, and of some of the many enzymes known to be required between the synthesis of the primary translation product and the secretion of the processed phosphorylated and glycosylated proteins (see Bergeron *et al.*, 1975; Palade, 1975; MacKinlay *et al.*, 1977; Keenan *et al.*, 1978; A. P. Boulton, C. D. Lane & R. K. Craig, unpublished work).

Although RNA-complexity analysis provides information about the presence or otherwise of specific poly(A)-containing RNA species within a given RNA population, it does not provide information which relates to the relative state of protein synthesis *in vivo*. This we can obtain from polyribosomes run-off *in vitro* in the presence of a suitable inhibitor of initiation. In these experiments, protein synthesis reflects only the elongation of nascent peptide chains that have been initiated *in vivo*. Consequently the identification of both α -lactalbumin and pre- α -lactalbumin in the run-off products of membrane-bound polyribosomes provides evidence that, in common with the immunoglobulin light chain (Blobel & Dobberstein, 1975a) and lysozyme (Palmiter *et al.*, 1978), the processing of pre- α -lactalbumin to α -lactalbumin *in vivo* occurs before the completion of the nascent polypeptide chain.

The situation regarding the caseins is less definitive with respect to the occurrence or otherwise of processing before the completion of the nascent peptide chain. However, the paradoxical distribution of [³⁵S]methionyl-peptides after run-off of membrane-bound polyribosomes, when compared with the distribution after run-off of free polyribosomes or mRNA-directed cell-free products, must reflect the real distribution of methionine residues within the nascent casein peptides at a moment in time *in vivo*.

Caseins A, B and C each contain one, two and one methionine residues respectively (Craig *et al.*, 1978). From the limited *N*-terminus sequence data that we have available, we know that casein B has an *N*-terminal methionine residue but no other methionine within 16 residues, whereas casein C has its only methionine residue at position 33 (R. K. Craig & J. I. Harris, unpublished work). Therefore if, as predicted by the 'signal hypothesis' (Blobel & Dobberstein, 1975a,b), the initiation of protein synthesis occurs in the free state, followed by considerable protein synthesis before the nascent 'signal' peptide is capable of locating on the presumptive membrane receptor sites, then it is reasonable to suppose that any methionine residues located towards the *N*-terminus of a secretory protein will be synthesized *in vivo* on free polyribosomes. Subsequent run-off *in vitro* of the resultant membrane-bound polyribosomes in the presence of [³⁵S]methionine will not involve the incorporation of label into these residues. Consequently, unless other methionine residues are

present, such peptides will be undetectable by radioautography when completed *in vitro*. Such an argument seems valid when applied to our own data. Thus for casein C, if we allow 15–20 residues (see Gaye *et al.*, 1977) for the 'signal' sequence, then provided that attachment occurs before a total of 48–53 residues have been synthesized, casein C should be fully labelled with [³⁵S]methionine at position 33 during run-off of membrane-bound polyribosomes *in vitro*. Conversely, if attachment has not occurred at this stage, then one would find no label in this position. As the relative degree of labelling of casein C is markedly diminished, it would be reasonable to argue that attachment may well occur close to the addition of residue 33 when the nascent peptide-chain length is in the order of 48–53 residues, an estimate very similar to the length reported in polyribosome run-off experiments *in vivo* (Palmiter *et al.*, 1977), and in synchronized mRNA-directed cell-free protein-synthesizing systems (Rothman & Lodish, 1977). If a similar argument is applied to α -lactalbumin (methionine at residue 90; Brew, 1972), then both attachment and processing of the 'signal' peptide must occur before the addition of this methionine residue. Moreover, it seems likely that the single methionine residue of casein A and the second methionine residue of casein B are also added after attachment and are consequently present in apparently enhanced amounts in relation to casein C on analysis of membrane-bound polyribosome run-off products. Conversely, these are relatively less intense when compared with casein C, when all are synthesized in a mRNA-directed wheat-germ cell-free protein-synthesizing system, or by free polyribosome run-off.

In addition to providing information about the timing of attachment and processing *in vivo*, the marked difference in the distribution of methionine residues, particularly the caseins, after run-off in the wheat-germ cell-free protein-synthesizing system suggests that the presence of free polyribosomes directing the synthesis of milk protein has not resulted from detachment of polyribosomes of membrane origin during preparation. Furthermore, the data are also consistent with the notion that, once initiation has occurred in the free state, followed by attachment to the endoplasmic reticulum, presumably through interaction of the nascent *N*-terminal peptides with putative membrane receptor sites, then no further initiation occurs until all the nascent chains have been completed, and the mRNA is released from the endoplasmic reticulum. If this was not the situation, the distinctive distribution of methionine residues would not be apparent.

Our results, although consistent with the initiation of milk-protein synthesis on free polyribosomes, also point to the occurrence of milk-protein synthesis on large free polyribosomes (Fig. 12). This suggests

that some milk-protein synthesis may go to completion in the free state, resulting in the miscompartmentation of new synthesized unprocessed milk proteins. These observations are similar to those of Houdebine (1977), who described the presence of 5% of casein polyribosomal mRNA on free polyribosomes in the lactating rabbit mammary gland, and up to 35% in the pregnant animal. We have described elsewhere (Craig *et al.*, 1979a) a selective proteolytic mechanism which prevents the accumulation within the cell of miscompartmentalized proteins that have resulted from heterotopic translation of proteins bearing *N*-terminal 'signal' sequences.

It seems likely that a similar mechanism may well function in the mammary gland, particularly in the pregnant animal, where it has been demonstrated in the mammary gland of the rat (Rosen *et al.*, 1978) and the rabbit (Shuster *et al.*, 1976; Houdebine, 1977) that milk-protein mRNA species are associated with polyribosomes and milk proteins are synthesized, but that secretory activity is low.

Our results concerning the subcellular distribution of milk-protein mRNA species are also in agreement with work on other normal tissue, which describe the association of 15% of rat liver α_{2u} -globulin mRNA with free polyribosomes (Kurtz *et al.*, 1978) and 2% of rat liver albumin mRNA with the free polyribosome fraction (Yap *et al.*, 1977), but the association of the bulk of the mRNA with the membrane-bound polyribosomes. This discrepancy between the precise proportion of each mRNA species associated with the free polyribosomes probably reflects the size of the proteins concerned, and therefore the percentage of the total peptide that must be synthesized before attachment to the membrane may occur. Thus one might expect a higher proportion of milk-protein (mol.wt. 14000–28000; Craig *et al.*, 1976) and α_{2u} -globulin (mol.wt. 26400; Roy, 1973) mRNA species associated with free polyribosomes than of those for serum albumin (mol.wt. 68000; Tanford *et al.*, 1967).

In addition to secretory proteins synthesized by the liver and mammary gland, considerable data have also accumulated about the intracellular site of synthesis of immunoglobulins. Unfortunately the situation here requires clarification, as some have reported the almost exclusive association of immunoglobulin synthesis with the membrane-bound polyribosomes (Cioli & Lennox, 1973; Blobel & Dobberstein, 1975a), whereas others have reported its synthesis on both free and membrane-bound polyribosomes (Lisowska-Bernstein *et al.*, 1970; Okuyama *et al.*, 1977). The interpretation of these observations is particularly difficult, as reports concern different mouse plasmacytoma cell lines.

We conclude the following from our studies on the intracellular distribution of polyribosomal poly(A)-containing RNA in the lactating guinea-pig mammary gland.

(i) As predicted by Siekevitz & Palade (1960), the primary site of milk-protein synthesis is the endoplasmic reticulum, though this is also the site of synthesis of a subset of proteins, the precise nature of which has yet to be determined.

(ii) The data are consistent with the notion that protein synthesis *in vivo* is initiated on free polyribosomes, resulting after the synthesis of 50–60 amino acids with attachment to the endoplasmic reticulum (casein C), presumably through interaction of the nascent *N*-terminal peptide with specific receptor sites. Furthermore, the 'signal' sequence if present may then be removed during on-going translation (α -lactalbumin), results in agreement with the central aspect of the 'signal hypothesis' (Milstein *et al.*, 1972; Blobel & Dobberstein, 1975a,b).

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