Isolation and characterization of casein mRNAs from lactating ewe mammary glands

P. Gaye and L.M.Houdebine

Laboratoire de Physiologie de la Lactation, Institut National de la Recherche Agronomique, C.N.R.Z., 78350 Jouy-en-Josas, France

Received 12 March 1975

SUMMARY

RNA from bound polysomes of lactating ewe's mammary gland directs the synthesis of the three major milk proteins (α_s , β and κ -caseins) in a cell-free system derived from rabbit reticulocytes. The "in vitro" product was identified by immunoprecipitation with specific antibodies and by electrophoresis in SDS polyacrylamide gel. Each of these messengers was purified from 20 to 25 fold from total membrane-bound polysomal RNA using poly U-Sepharose chromatography. This purified fraction assayed in a reticulocyte cell-free system is able to direct also the synthesis of 2 minor secretory proteins (β -lactoglobulin and α -lactalbumin). The messenger RNAs purified by hybridization to poly U-Sepharose have a sedimentation coefficient of about 12 S and an apparent molecular weight of approximately 3.5×10^5 daltons was observed by polyacrylamide gel electrophoresis under denaturing conditions. This value which correspond to about 900 nucleotides is significantly greater than the number expected for coding milk proteins.

INTRODUCTION

The induction of milk protein synthesis (α , β , κ -caseins, β -lactoglobulin, α -lactalbumin) occurs naturally during late pregnancy and, under experimental conditions, after the action on a well developed mammary gland of the species specific lactogenic hormonal complex¹.

The study of messenger RNAs provides an approach to the investigation of gene expression and regulation of specific milk protein synthesis in mammary cells.

During the lactating period the major activity of protein synthesis of mammary cells is focused on milk proteins which appear to be preferentially synthesized on polyribosomes attached to the reticulum membranes^{2,3,4,5}.

We recently reported^{6,7} that mRNA fractions isolated from membrane-bound polysomes by sucrose gradient or poly U-Sepharose chromatography is able to direct the synthesis of α_s -casein in a rabbit reticulocyte cell-free system. CAMPBELL et

al.⁸ reported equally that the mRNA for α -lactalbumin was present in polysomal RNA isolated from lactating guinea-pig mammary glands.

The present report extends our previous investigations⁹ and shows that casein messengers, which are the major components of the mRNAs for secretory proteins in the lactating mammary gland, can be assayed quantitatively, using a cell-free protein synthesizing system. These mRNAs bind selectively to poly U-Sepharose indicating that they contain poly A-rich segments. The molecular weight of these mRNAs was about 3.5×10^5 daltons, as determined by polyacrylamide gel electrophoresis and sucrose gradient centrifugation under denaturing conditions. In addition we demonstrate that this mRNA fraction contains the information for coding two minor secretory proteins (β -lactoglobulin and α -lactalbumin).

The ability of these RNAs to promote the "in vitro" synthesis of β -lactoglobulin and α -lactalbumin was also examined.

MATERIALS AND METHODS

Mammary glands were taken from ewes of the "Préalpes du Sud" breed between the 30th and the 50th days of lactation. In order to eliminate the milk contained in the gland, the ewes were hand milked after intravenous injections of oxytocin shortly before slaughter.

Preparation of membrane-bound polysomes and RNA

Membrane-bound polysomes were prepared from the 40 000 g microsomal pellet as described previously⁷.

RNA was extracted from polysomes with phenol at pH 9¹⁰ chloroform was added to the phenol at the last extraction step. Before being translated the RNA fractions were resuspended in 3M Na acetate, 5 mM EDTA pH 6 and centrifuged 15 min. at 20 000 g. This treatment removed some impurities and allowed a more efficient translation of the mRNA in the reticulocyte lysate¹¹. All the RNA fractions were precipitated by ethanol and stored at -20°C until use.

Purification of mRNA

a) Zonal centrifugation

RNAs (800-1500 A₂₆₀) extracted from membrane-bound

polysomes by SDS alone or SDS-phenol were fractionated in a Beckman TiXIV rotor on an exponential 8-28 % sucrose gradient. The sample was layered on the gradient in 20 ml of 4 % sucrose and overlaid by 120 ml buffer. All sucrose solutions were in 50 mM Tris, 2 mM EDTA, 0.2 % SDS, 0.1 % DOC.

b) Affinity chromatography

The purification of poly A-containing RNAs from polysomal RNA was carried out by poly U-Sepharose chromatography. Poly U (Miles) was bound to activated Sepharose (Sephadex, Uppsala) by the 5'P end as described by WAGNER et al.¹². Polysomal RNA was applied to the column in TNES buffer (50 mM Tris, pH 7.5, 0.12 M NaCl, 0.5 % SDS). Poly A-containing RNAs were eluted by 0.05 % SDS in water according to FIRTEL et al.¹³. The eluate was adjusted to 0.12 M NaCl and immediately rechromatographed on the column reequilibrated with TNES buffer. This second chromatographic step was required for complete removal of ribosomal RNA.

Polyacrylamide gel electrophoresis of RNA

This technique was used under various conditions for the determination of mRNA molecular weight.

1) Polyacrylamide gel with uniform acrylamide concentration (2.4 %) in phosphate-SDS-buffer as described by LOENING¹⁴.

2) Polyacrylamide gel with exponential concentration (2.3 , 13 %) in phosphate buffer as described by MIRAULT and SCHERRER¹⁵.

3) Polyacrylamide gel with uniform acrylamide concentration (2.6 %) in 99 % formamide, 20 mM NaCl as described by STAYNOV et al.¹⁶.

Acrylamide was polymerized in a quartz tube (6 mm internal diameter). At the end of the run, the gels were directly scanned in a Joyce LoebI apparatus at 265 nm.

Assay for mRNA activity

Polysomal RNA was isolated and washed as described above. The sample containing between 10 and 30 µg of polysomal RNA or 0.5 to 2 µg of purified mRNA was assayed as described previously⁷ using lysates from rabbit reticulocytes.

Radioactive isoleucine and proline were used to follow casein synthesis, since the level of these amino acids was

higher in caseins (28 to 43 residues)¹⁷, than in globin (5 to 10 residues)¹⁸.

The incorporation of ¹⁴C isoleucine and ¹⁴C proline into various milk proteins was determined by immunoprecipitation with specific antiserum according to the procedure described by RHOADS et al.¹⁹ and PALMITER¹¹. The background radioactivity in the immunoprecipitates of the control was about 0.1% of globin synthesis (ranging from 350 to 600 cpm).

Preparation of antiserum

Ovine caseins α_s and κ were utilized for the preparation of immunserum, the β -casein was of bovine origin.

The antiserum to purified α_s , β and κ -caseins was produced in the guinea-pig and in the rabbit by a serie of five injections in complete Freund's adjuvant. The antisera for each type of casein were tested for their specificity by the Outcherlony immuno diffusion method.

The antisera to purified bovine β -lactoglobulin and α -lactalbumin were obtained from Antibodies Incorporated. These two proteins have the same antigenic properties as ewe's milk proteins^{20,21}.

Polyacrylamide gel electrophoresis of proteins

The immunoprecipitate was dissolved in a mixture containing : 10 mM phosphate, pH 7.1 ; 2 % SDS ; 4 M urea ; 5 % β -mercaptoethanol and 10 % glycerol, treated 2 min. at 100°C and subjected to electrophoresis on 10 % acrylamide gel according to WEBER and OSBORN²².

RESULTS

Purification of casein mRNA

The purification of casein mRNA was conducted by two independent methods.

Zonal centrifugation

RNAs (1000 A₂₆₀) extracted from membrane-bound polyosomes by SDS or SDS-phenol were fractionnated by zonal centrifugation (fig. 1). The 7-17 S fraction between the arrows represent 5 to 5.5 % of the RNAs applied on the gradient. Polyacrylamide gel electrophoresis revealed that this fraction con-

Figure 1 - Zonal centrifugation profile of RNA from membrane-bound polysomes. Centrifugation was performed at 45 000 rpm for 16 hours, at 4°C in a Beckman T_iXIV rotor.

tains RNAs smaller than 18 S RNA (data not shown). Although this fraction was only slightly contaminated with 18 S RNA only 16.5 % of the RNA could be hybridized to the poly U-Sepharose column. This quantity corresponds to about 0.8 % of the RNA originally applied to the gradient which agrees with the yield of the poly A-containing RNA isolated directly from total bound polysomal RNAs, suggesting that most of the mRNAs were recovered in the 7-17 S fraction. If higher amounts of RNA (e.g. 1500 A₂₆₀) were applied to the gradient, the separations obtained were of lesser quality, owing to some trailing of mRNAs. It has been demonstrated that half of mRNAs for casein are devoid of poly A sequence⁷, hence about 70 % of the 7-17 S fraction are likely not mRNAs. These contaminating RNAs could also be observed with RNAs extracted from mammary gland free polysomes and rabbit reticulocyte polysomes. The amount of these contaminating RNA_s was related to the proportion of monomer ribosomes in polysomal preparations. Similar observations have been reported by LANYON et al.²³ in reticulocytes and PIPERNO et al.²⁴ in calf lens.

Poly U-Sepharose chromatography

Poly A-containing RNAs isolated by poly U-Sepharose chromatography are completely devoid of rRNA as shown in figure 2. The mRNA fraction obtained represented about 0.8-0.9 % of the polysomal RNA applied to the column, in good agreement with the calculation performed above. However this technique did not enable us to isolate all the mRNAs for caseins since about 50 % of messenger activity proved not to contain the poly A sequence⁷. When nitro cellulose filters¹⁰, cellulose²⁵ or oligo dT cellulose were used, lower yields of mRNA were obtained.

Figure 2 - Polyacrylamide gel electrophoresis of poly A-containing RNA. mRNA (1.5 A₂₆₀) was dissolved in phosphate-SDS buffer and submitted to electrophoresis in exponential gel (2.3 to 13 %) for 6 hours at 5 mA/gel.

———— mammary gland mRNA
 ----- rabbit reticulocyte mRNA

Measurement of messenger activity

The total membrane-bound polysomal RNA and the RNA, obtained by zonal centrifugation or poly U-Sepharose chromatography were tested for their ability to direct the synthesis of caseins, "in vitro" in a reticulocyte lysate. The determination of the synthesis of α_s , β and κ -caseins after addition of the total bound polysomal RNA to a reticulocyte lysate is shown in figure 3 a. In all cases there was a linear increase in the amount of specific products synthesized with the lower concen-

tration of RNA but at higher concentrations the response was not proportional.

Figure 3 - α_S , β and κ -caseins synthesis after addition of polyosomal RNA (a) or purified mRNA (b). Varying amounts of RNA were added to a reticulocyte lysate and incubated for 90 min. at 27°C. Aliquots (50 to 200 μl) of the reaction mixture were used to determine by immunoprecipitation the amount of each protein synthesized. All values refer to the radioactivity in 250 μl of lysate.

Table I
SYNTHESIS OF CASEINS IN A RABBIT RETICULOCYTE LYSATE
PROGRAMMED WITH RNA ISOLATED FROM MEMBRANE-BOUND POLYSOMES

Sample	α_S -casein			β -casein			κ -casein		
	cpm/ μg RNA	pmoles/ μg RNA *	pmoles % **	cpm/ μg RNA	pmoles/ μg RNA	pmoles %	cpm/ μg RNA	pmoles/ μg RNA	pmoles %
1	1 320	0.224	41.90	1 950	0.216	40.30	600	0.95	17.80
2	1 000	0.170	-	1 600	0.177	-	-	-	-
3	1 300	0.221	42.75	1 850	0.205	39.65	575	0.91	17.60
4	1 250	0.212	41.80	1 900	0.210	41.40	500	0.85	16.80

* Calculated from the specific activity of ^{14}C isoleucine and ^{14}C proline (1 picomole = 210 cpm) and the number of isoleucine and proline residues in each protein: α_S -casein 28 residues; β -casein 43 residues; κ -casein 30 residues (assuming that the number of proline and isoleucine residues in ewe casein are identical to bovine casein).

** Calculated from the sum of three caseins.

On table I, we summarized the data obtained with different samples of total bound polysomal RNA. The specific activity for α_S , β and κ -caseins of different samples of polysomal RNA examined was variable but the relative proportion of each

casein synthesized was nearly constant. These results suggest that the mRNA_s specific for each casein are present in constant ratios in membrane-bound polysomes isolated from lactating mammary glands.

The ability of the mRNA fraction (7-17 S) isolated by zonal centrifugation to direct "in vitro" the synthesis of α_s -casein was examined independently. In these experiments, we observed a 3 to 4 fold purification in messenger specific activity for α_s -casein with respect to unfractionated polysomal RNA.

The activity of mRNA fractions purified from total membrane-bound polysomal RNA by hybridization to poly U-Sepharose tested in the reticulocyte system are proportional to concentrations (between 0.5 and 2 μ g of mRNA - fig. 3 b). At the highest RNA concentration the response reached a plateau and even the synthesis of globin is notably depressed (results not shown) suggesting that the added mRNA competes with globin mRNA at the translational level.

The results of table II show that the degree of purification achieved for each of these messengers resulted in a 20 to 25 fold increase in specific activity. The fact that all 3 messengers were purified to nearly the same extent suggests that the percentage of poly A-linked molecules hybridizing with poly U-Sepharose is identical for all the messengers.

Table II
SYNTHESIS OF CASEIN IN A RABBIT RETICULOCYTE LYSATE PROGRAMMED
WITH mRNA ISOLATED BY POLY U-SEPHAROSE CHROMATOGRAPHY
FROM TOTAL POLYSOMAL RNA

Sample	α_s -casein			β -casein			κ -casein		
	cpm/ μ g RNA	pmoles/ μ g RNA *	pmoles % **	cpm/ μ g RNA	pmoles/ μ g RNA	pmoles %	cpm/ μ g RNA	pmoles/ μ g RNA	pmoles %
1	25 500	4.33	41.00	38 500	4.26	40.30	12 500	1.98	18.70
2	28 000	4.76	41.15	40 000	4.43	38.30	15 000	2.38	20.55
3	26 500	4.50	41.55	37 000	4.10	37.85	14 100	2.23	20.60

* Calculated from the specific activity of 14 C isoleucine and 14 C proline (1 picomole = 210 cpm) and the number of isoleucine and proline residues in each protein: α_s -casein 28 residues; β -casein 43 residues; κ -casein 30 residues (assuming that the number of proline and isoleucine residues in ewe casein are identical to bovine casein).

** Calculated from the sum of three caseins.

The values obtained in two different experiments were respectively (3 000, 3 200 cpm per μg of RNA) for β -lactoglobulin and (950, 1 500 cpm per μg of RNA) for α -lactalbumin. Assuming that β -lactoglobulin and α -lactalbumin have respectively 17 and 11 isoleucine and proline residues per chain, the number of β -lactoglobulin and α -lactalbumin molecules synthesized represents respectively 9 and 5 % of the values obtained for the 3 caseins.

Characterization of the immunoprecipitable "in vitro" products

Figure 4 - Comparison by SDS acrylamide gel electrophoresis of ^{14}C casein and ^{14}C β -lactoglobulin synthesized in reticulocyte lysates after addition of mRNA with casein and β -lactoglobulin labelled in mammary explants incubated with ^3H amino acids.

The explants were labelled during two hours with ^3H isoleucine and proline, an homogenate was prepared and each protein was isolated by immunoprecipitation with a specific antiserum as described under "methods". The immunoprecipitate ^3H and ^{14}C were solubilized by heating in the presence of 2 % SDS and β -mercaptoethanol, combined and subjected to electrophoresis. The direction of migration is from left to right.

a) α_s -casein ; b) β -casein ;
c) κ -casein ; d) β -lactoglobulin

In order to examine the nature of the radioactivity precipitated from the lysate by each antibody the immunoprecipitate was dissociated by SDS and subjected to polyacrylamide gel electrophoresis in SDS buffer.

Figure 4 shows that the radioactivity in α_s -casein, β -casein, κ -casein and β -lactoglobulin immunoprecipitated when subjected to SDS acrylamide gel electrophoresis, migrated as a single peak which nearly coincided with protein synthesized by mammary explants incubated in the presence of ^3H isoleucine and ^3H proline.

α_s -casein and β -casein migrated as a single peak with an apparent molecular weight of about 25 000 daltons. A symmetrical peak was observed in the 20 000 and 18 000 molecular weight region for κ -casein and β -lactoglobulin. Furthermore we observed in the gels the presence of a small amount of low molecular weight labeled polypeptidic components, which were immunoprecipitated and may represent released incomplete fragments of these different milk proteins.

Determination of the molecular weight of mRNA_s

Only poly A-containing RNAs were used for this purpose, since this fraction only had sufficient purity. Indeed the 7-17 S fraction isolated by zonal centrifugation was so heavily contaminated that its profile on polyacrylamide did not vary significantly after removal of the poly A-containing messengers through chromatography on poly U-Sepharose.

Electrophoresis of mRNA on exponential polyacrylamide gel in phosphate buffer (fig. 2) indicated that this fraction was heterogeneous with two major peaks corresponding to about 12 S and 17 S when compared to 28 S and 18 S ribosomal RNA and to 9 S globin mRNA_s isolated under the same conditions from rabbit reticulocytes. These values were higher than expected for the synthesis of milk proteins (molecular weight 18 000 - 25 000). Sucrose gradient centrifugation in Tris-EDTA-SDS buffer suggested that the mean sedimentation coefficient of this mRNA fraction was probably not higher than 12 S⁹.

The possibility of an artefact in polyacrylamide gel must be considered, since the poly A sequence proved to reduce the mobility of poly A-containing RNAs²⁶. However, an overnight

extended electrophoresis in this exponential gel led to the same mRNA profile although after too long a run all the RNAs had certainly reached the acrylamide concentration which did not allow any further migration. A similar molecular weight estimation was obtained when electrophoresis was carried out with gels having a constant acrylamide concentration (2.4 %).

The possibility of aggregate formation was partly eliminated by treatment of mRNAs with 85 % DMSO at 37°C for 15 minutes just before electrophoresis : a very similar mRNA profile was obtained (data not shown).

In order to reduce as much as possible the influence of the mRNAs secondary structure, polyacrylamide gel electrophoresis and sucrose gradient centrifugation were even performed in denaturing solvents. Polyacrylamide gel electrophoresis in formamide 99 % NaCl 20 mM revealed that the sedimentation coefficient of mRNAs ranged between 9.5 and 12.5 S when compared to 28 S, 18 S, 5 S and 4 S RNA (fig. 5).

Figure 5 - Polyacrylamide gel electrophoresis of poly A-containing mRNA in formamide.

mRNA (1.5 A₂₆₀) was dissolved in 99 % formamide 20 mM NaCl and submitted to electrophoresis in the same buffer for 4 hours at 5 mA/gel.

The denaturing action of formaldehyde has been demonstrated by BOEDTKER²⁷. The mRNAs were treated by 3 % HCHO in phosphate buffer and then centrifuged on a sucrose gradient in triethanolamine containing 3 % HCHO. Results of figure 6 indicated that the mean sedimentation coefficient was not higher

that 12 S. This latter value must be accepted and not the value resulting from polyacrylamide gel electrophoresis in phosphate buffer. The molecular weight of mRNAs so estimated corresponds to about 900 nucleotides, a value significantly greater than the number required for the synthesis of caseins.

Figure 6 - Sedimentation profile of poly A-containing RNA in formaldehyde sucrose gradient.

mRNA (5 A₂₆₀) was dissolved in 90 mM Na₂ H PO₄ and treated for 15 min. at 63°C, then chilled and layered over 18 ml 5-20 % sucrose gradient in 50 mM triethanolamine pH 7.5, 0.05 % SDS, 0.025 % DOC, 3 % formaldehyde.

Gradients were run in SW27 for 44 hours at 25 000 rpm, temperature 4°C.

———— mammary gland mRNA
 ----- rabbit reticulocyte mRNA

CONCLUSION

The results reported here demonstrate that the mRNA isolated from bound polysomes of the ewe lactating mammary gland is able to direct the synthesis of caseins α_s , β and κ in a cell-free system derived from rabbit reticulocytes.

Assuming that the amino acid composition of ewe caseins

is similar to bovine caseins, the relative amounts of these molecules synthesized in a reticulocyte cell free system in response to polysomal mammary mRNA correspond to about 42, 38 and 20 % respectively for α_s , β and κ -casein. These proportions are of the same order as their ratio in total ewe casein (α_s -casein 48 %, β -casein 36 % and κ -casein 16 %) determined by carboxyl terminal amino acid analysis (RIBADEAU-DUMAS, personal communication).

These results indicate that the translation of mRNA_s to caseins is accomplished with a high degree of fidelity in a reticulocyte lysate, without any requirement for additional components from the mammary gland. Several mRNA species have been translated in reticulocyte systems^{19,28,29,30,31,32,33,34,35,36} and in no case was there any evidence that this system is mRNA specific. In addition, PALMITER¹¹ has shown recently that an ovalbumin mRNA is translated in a reticulocyte lysate with nearly the same rate of chain initiation and elongation as obtains in the intact hen oviduct.

Using poly U-Sepharose chromatography we were able to obtain the RNA containing the poly A sequences. The mRNAs account for 0.8 to 1 % of the total membrane-bound polysomal RNA. This method gave a 20 to 25 fold purification in messenger specific activity for α_s , β and κ -caseins as compared to unfractionated polysomal RNA. In addition this purified fraction is able to direct "in vitro" the synthesis of β -lactoglobulin and α -lactalbumin. The fact that the purification of all casein mRNA_s was achieved to a similar degree by poly U-Sepharose chromatography suggests a relatively identical extent of heterogeneity in poly A content in α_s , β and κ -casein mRNAs and supports the hypothesis that the poly A segments of these three mRNAs are degraded in a similar manner.

To give further support to the identification of proteins synthesized "in vitro", we analysed the immunoprecipitated products by SDS acrylamide gel electrophoresis, allowing the comparison of molecular weight of the polypeptides synthesized in a cell free system with proteins, isolated from mammary explants incubated "in vitro". In the course of these studies, no large molecular weight precursor molecules for caseins were found and this fact does not agree with the hypothesis recently formu-

lated ³⁷ that caseins being initially synthesized as one giant macromolecule are then transformed into the casein monomers usually found by a specific protease. However the present results cannot formally exclude the possibility that caseins are first synthesized as slightly larger precursors, as demonstrated for immunoglobulins ^{32,38} the techniques used being not sufficiently sensitive to detect small variations in molecular size.

The molecular weight of casein mRNA purified by poly U-Sepharose chromatography can be estimated by polyacrylamide gel electrophoresis and sucrose gradient centrifugation under denaturing conditions. Taking values of 6.7×10^5 for the molecular weight of 18 S RNA ³⁹, values of 3.5×10^5 were estimated for casein mRNA. From this molecular weight the number of nucleotides calculated was about 900. This number is more than required for the amino acid sequence of caseins. Assuming that caseins α_s , β , κ have respectively 199, 209 and 169 amino acids ¹⁷, only about 600 nucleotides are required. The mRNA_s found are nearly 1.5 times this length and even when excluding 130 residues in poly A ⁷, there appear to be about 170 nucleotides more than would be necessary to encode for one casein molecule.

The presence of this untranslated sequence in the casein mRNA does not seem specific for these molecules, but appears now as a general characteristic of eukaryotic messengers ^{32,38,40,41,42,43}. The precise role of these untranslated sequences is still unknown, but they might be required for various control processes in the cell ^{44,45}.

It should now be possible to examine the mechanism by which the lactogenic hormones regulate the synthesis of each of these milk protein messengers and to characterize nuclear events related to the transcription of casein mRNAs.

ACKNOWLEDGEMENTS

We thank Miss Solange SOULIER for a gift of the purified ovine α_s and κ -casein and Dr Bruno RIBADEAU-DUMAS for a gift of bovine β -casein.

We are extremely grateful to Professor Hubert CLAUSER for his critical comment of the manuscript.

We acknowledge the excellent technical assistance of Mrs Danielle DROUET and Claudine PUISSANT.

This work was supported by grant n° 74.7.0172 from the
Délégation Générale à la Recherche Scientifique et Technique.

REFERENCES

1. Denamur, R. (1971) J. Dairy Res. 38, 237-264.
2. Brew, K. and Campbell, P.N. (1967) Biochem. J. 102, 265-274.
3. Beitz, D.C., Mohrenweiser, H.W., Thomas, J.W. and Wood, W.A. (1969) Arch. Biochem. Biophys. 132, 210.
4. Gaye, P. and Denamur, R. (1970) Biochem. Biophys. Res. Commun. 41, 266-272.
5. Gaye, P., Viennot, N. and Denamur, R. (1972) Biochim. Biophys. Acta 262, 371-380.
6. Gaye, P., Houdebine, L.M. and Denamur, R. (1973) Biochem. Biophys. Res. Commun. 51, 637-644.
7. Houdebine, L.M., Gaye, P. and Favre, A. (1974) Nucleic Acids Res. 1, 413-426.
8. Campbell, P.N., McIlreavy, D. and Tarin, D. (1973) Biochem. J. 134, 345-348.
9. Gaye, P., Houdebine, L.M., Pétrissant, G. and Denamur, R. (1973) Acta Endocrinologica, pp. 426-447, VIth Karolinska Symposium in Research Methods in Reproductive Endocrinology.
10. Lee, S.Y., Neudecki, J. and Brawerman, G. (1971) Proc. Nat. Acad. Sci. USA 68, 1331-1335.
11. Palmiter, R.D. (1973) J. Biol. Chem. 248, 2095-2106.
12. Wagner, A.F., Bugranesi, R.L. and SHEN, T.Y. (1971) Biochem. Biophys. Res. Commun. 45, 184-189.
13. Firtel, R.A., Jacobson, A. and Lodish, H.F. (1972) Nature N.B. 239, 225-228.
14. Loening, U.E. (1964) Biochem. J. 113, 131-138.
15. Mirault, M.E., Scherrer, K. (1971) Europ. J. Biochem. 23, 372-386.
16. Staynov, D.Z., Pinder, J.C. and Gratzner, W.B. (1972) Nature N.B. 235, 108-110.
17. Mercier, J.C., Grosclaude, F. and Ribadeau-Dumas, B. (1973) Milchwissenschaft 27, 401-408.
18. Dayhoff, M.O. (1969) Atlas of Protein sequence and Structure, D42-D54.
19. Rhoads, R.E., McKnight, G.S. and Schimke, R.T. (1971) J. Biol. Chem. 246, 7407-7410.
20. Johke, T., Hageman, E.C. and Larson, B.L. (1964) J. Dairy Sci. 47, 28-31.
21. Tananashi, N., Brodbeck, U. and Ebner, K.E. (1968) Biochim. Biophys. Acta 154, 247-249.
22. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406.
23. Lanyon, W.G., Paul, J. and Williamson, R. (1972) Europ. J. Biochem. 31, 38-43.
24. Piperno, G., Bertazzoni, U., Berns, A.J.B. and Bloemendal, H. (1974) Nucleic Acids Res. 1, 245-255.
25. Kitos, P.A., Saxon, G. and Amos, H. (1972) Biochem. Biophys. Res. Commun. 47, 1428-1437.
26. Armstrong, J.A., Edmonds, M., Nakazato, H., Phillips, B.A. and Vaughan, M. (1972) Science 176, 526-528.
27. Boedtker, H. (1971) Biochim. Biophys. Acta 240, 448-453.

28. Lockard, R.E. and Lingrel, J.B. (1969) Biochem. Biophys. Res. Commun. 37, 204-212.
29. Means, A.R., Comstock, J.P., Rosenfeld, G.C. and O'Malley, B.W. (1972) Proc. Nat. Acad. Sci. USA 69, 1146-1150.
30. Stavnezer, J. and Huang, R.C.C. (1971) Nature 230, 172-176.
31. Milstein, C., Brownlee, C.G., Harrison, J.M. and Matthews, M.B. (1972) Nature N.B. 239, 117-120.
32. Mach, B., Faust, C. and Vassili, P. (1973) Proc. Nat. Acad. Sci. USA 70, 451-455.
33. Berns, A.J.M., Shons, G.S.A.M. and Bloemendal, H. (1972) Nature N.B. 236, 7-10.
34. Gallwitz, D. and Breindl, M. (1972) Biochem. Biophys. Res. Commun. 47, 1106-1111.
35. Palmiter, R.D. and Smith, L.T. (1973) Nature N.B. 246, 74-76.
36. Taylor, J.M. and Schimke, R.T. (1973) J. Biol. Chem. 248, 7661-7668.
37. Carroll, R.J., Thompson, M.P. and Farrell, H.M. Jr (1970). 28th A. Proc. Electron Microscope Society of America, p. 150.
38. Swan, D., Aviv, H. and Leder, P. (1972) Proc. Nat. Acad. Sci. USA 69, 1967-1971.
39. McConkey, E.H. and Hopkins, J.W. (1969) J. Mol. Biol. 39, 545-550.
40. Gaskill, P. and Kabat, D. (1971) Proc. Nat. Acad. Sci. USA 68, 72-75.
41. Berns, T.J.M., Schrens, V.V.A.M., Van Kraaikamp, M.W.G. and Bloemendal, H. (1973) Europ. J. Biochem. 33, 551-557.
42. Brownlee, C.G., Gartwright, E.M., Cowan, N.J., Jarvis, J.M. and Milstein, C. (1973) Nature N.B. 244, 236-240.
43. Haines, M.E., Carey, N.A., Palmiter, R.D. (1974) Europ. J. Biochem. 13, 549-560.
44. Steitz, J.A. (1969) Nature 224, 957-960.
45. Cory, S., Spahr, P.F. and Adams, J.M. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 1-10.