ISOLATION OF ACTIVE MESSENGER RNA FOR α_s CASEIN FROM BOUND POLYRIBOSOMES OF MAMMARY GLAND

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SUMMARY - RNAs have been extracted from mammary bound polyribosomes and purified by sucrose gradient centrifugation. A cell free mammary system (homologous, except for the addition of initiation factors of reticulocyte origin) and a lysate of reticulocytes, have been used to assay biologically active messenger RNA. One of these messenger RNAs directs the synthesis of a product with electrophoretic and immunological properties analogous to α_s casein.

Previous studies from this laboratory have shown that the specific milk proteins, α lactalbumin and β lactoglobulin, are preferentially synthetized by polyribosomes bound to the membrane of the endoplasmic reticulum of the mammary gland (1, 2). The present communication reports evidence for the isolation, from bound polyribosomes of the ewe mammary gland, of a RNA fraction having messenger properties for the milk protein α_s casein. The translation of RNAs was achieved in two ways, (a) by the use of a cell free system of ovine mammary gland, homologous except for the addition of initiation factors of reticulocyte origin, (b) by using a lysate of reticulocytes.

I - MATERIALS AND METHODS

The radioactive chemicals leucine ^{14}C (153 μC/μM), leucine ^{3}H (5 C/mM), isoleucine ^{14}C (131 μC/μM), proline ^{14}C (152 μC/μM) were supplied by C.E.A. (Saclay, France). Sucrose (RNAse free) was purchased from Schwartz Mann.

- Isolation of "messenger RNA" fraction

Bound polyribosomes were prepared from ewe mammary gland according to methods previously described (1, 2). The RNAs were extracted by SDS (0.5 % at 37°C for 5 min.) and then fractionated by centrifugation (Spinco rotor 42 for 17 h at 34 000 rpm) in an exponential sucrose gradient (15-35 % with SDS buffer). Sedimentation profiles were recorded at 254 nm using an Isco Recorder type UA-4. The fractions constituting the shoulder in the descending part of the

18 S RNA peak were then taken to a final concentration of 0.3 M Na Cl, pooled and precipitated at - 20 C with 2.5 volumes of 95° ethanol. The precipitate was redissolved in SDS buffer and centrifuged in an exponential sucrose gradient of 15 to 35 % (Spinco rotor SW 25 for 40 h at 25 000 rpm, 22°C). The RNA fractions isolated by the two successive centrifugations were then analysed by exponential polyacrylamide gel electrophoresis in a SDS medium according to the method of MIRAULT and SCHERRER (1971).

- Protein synthesis in cell-free system

. The cell free mammary gland system was constituted of ribosomal sub-units (FALVEY and STAEHELIN, 1970), dialysed postribosomal supernatant, t.RNAs, and initiation factors prepared by extracting reticulocyte ribosomes with 0.5 M KCl (GILBERT and ANDERSON, 1970). The incubation medium used consisted of the following constituents in a final volume of 150 µl : Tris HCl pH 7.4, 20 mM ; KCl, 90 mM ; $Mg\ Cl_2$, 3.5 mM ; ATP, 1 mM ; GTP, 0.2 mM ; PEP, 3 mM ; pyruvate kinase 0.1 IU ; DTT, 1 mM ; 19 ^{12}C amino-acids, 50 µM ; ^{14}C leucine, 0.5 µCi ; 0.18 O.D. of mammary t.RNA ; postribosomal supernatant, 300 µg ; ribosomal sub-units, 0.6 O.D. ; 0.5 M KCl wash fraction from rabbit reticulocyte ribosomes, 200 µg. The incubation was carried out at 37°C in the presence or in the absence of bound polyribosome "messenger RNA fraction" which was added in varying concentrations as described in the "Results section". The proteins synthesized by this procedure were finally recovered by adding 2 ml of 10 % TCA, heating to 90°C for 15 min. and collecting over glass filter.

. Reticulocyte lysates were prepared and used according to the procedures of LINGREL (1972).

Radioactivity was determined by liquid scintillation spectrometry (Spectrometer Packard 3375).

- Characterization of synthesized polypeptides

Electrophoresis of polypeptides synthesized in vitro was carried out on polyacrylamide gel in the presence of SDS (LAEMMLI, 1970), using 15 % acrylamide in the running gel and 3 % in the stacking gel. The proteins had been previously dissociated at 100°C for 2 min. by a solution containing Tris 0.125 M pH 6.8 ; SDS 2 % ; mercaptoethanol, 5 % ; glycerol, 15 % ; bromophenol blue, 0.01 %.

Antibodies to α_s casein were prepared from purified ovine α_s casein injected into guinea-pigs (5 injections). Immuno-precipita-

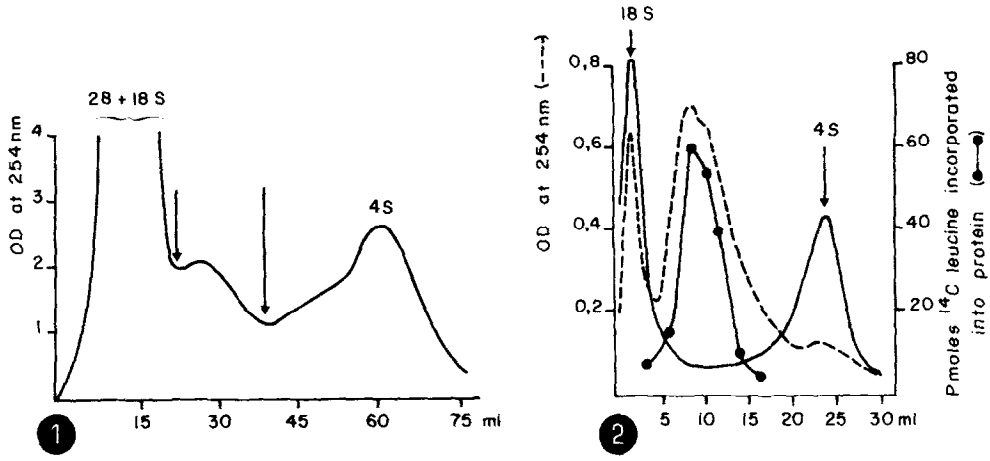

Fig. 1 - Sedimentation profile of polyribosomal RNA extracted from ewe mammary gland polyribosomes by SDS. Centrifugation was performed for 17 h at 34 000 rpm and at 22°C in an exponential sucrose gradient (15-35 %) in SDS buffer in polycarbonate tube of rotor 42.

Fig. 2 - Sedimentation profile obtained after a second centrifugation of pooled material from fractions between the arrows of Fig. 1

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tion and purification of the in vitro synthesized proteins was carried out according to the method of PALMITER & al. (1972) for ovalbumin. The resulting antigen-antibody complexes were dissociated and submitted to polyacrylamide gel electrophoresis as described above.

II - RESULTS

- Isolation of a RNA fraction from bound polyribosomes

Preparative centrifugation on sucrose gradient of polyribosomal RNA revealed the existence of RNAs which sedimented more slowly than RNA 18 S and gave rise to a well marked shoulder towards the base of the 18 S peak (Fig. 1). The fractions which were situated between the arrows in figure 1 were pooled and submitted to a second centrifugation in a sucrose gradient using a Spinco rotor SW 25 for 40 h at 25 000 rpm at 22°C. This procedure showed up an asymmetrical peak between 7 S and 17 S (Fig. 2). These RNA fractions possessed sedimentation characteristics compatible with the molecular weights of messenger RNAs responsible for the synthesis of milk proteins (M.W. 15 - 25 000). They represent, after two centrifugations, from

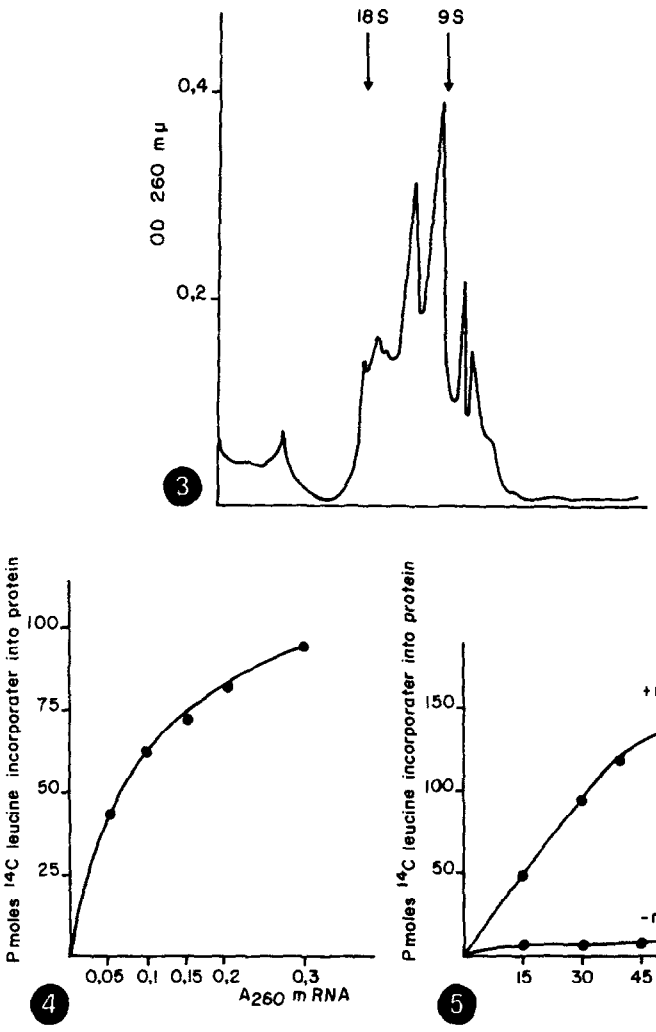

Fig. 3 - Electrophoretic analysis of RNA fraction from second centrifugation in sucrose gradient. The electrophoresis was conducted on a 2.5-13 % exponential polyacrylamide gel at 100 V during 6 h. The arrows indicate the position of 18 S and 9 S globin RNA.

Fig. 4 - Time course of exogenous mammary gland messenger RNA directed protein synthesis on mammary gland ribosomal subunits in a cell free system.

Fig. 5 - Incorporation of ^{14}C leucine into protein as a function of mammary messenger RNA concentration.

0.5 to 0.6 % of the RNA contained in the bound polyribosomes. Analysis of these RNA fractions by polyacrylamide gel electrophoresis with SDS indicated the presence of different molecular species, most of which were probably not messenger RNAs (Fig. 3).

- Evidence for messenger activity in the RNA fraction isolated by centrifugation in sucrose gradient

The RNA fraction was added to a cell free mammary system which was characterized by a very low endogenous synthetic activity, even after the addition of reticulocyte initiation factors (Fig. 4). There was a large increase in the incorporation of ^{14}C leucine into protein (Fig. 4), and the resulting stimulation depended on the amount of RNAs introduced into the medium (Fig. 5). It is noteworthy that protein synthesis by this cell free system was equally dependent on the presence of reticulocyte initiation factors : in their absence, "messenger RNAs" were devoid of activity. When messenger activities of the various messenger RNA fractions constituting the asymmetrical peak separated by the second centrifugation were plotted against absorbance at 254 nm, evidence of a close correlation was obtained (Fig. 2).

- Characterization of the proteins synthesized by "messenger RNA" isolated from bound polyribosomes

The various proteins directed by "messenger RNAs" added to the cell free mammary system were separated by electrophoresis on polyacrylamide gel in the presence of SDS ; there resulted a number of peaks of radioactivity. However the major peak migrated at the same rate as α_s casein purified from ovine milk (Fig. 6). The other radioactive components also showed electrophoretic behaviour compatible with the molecular weight of the principal milk proteins. In the absence of exogenous RNA, protein synthesis was very greatly reduced, and only polypeptides of low molecular weight were obtained.

In a separate experiment, 9 S messenger RNA for globin was added to the cell free mammary system used in the preceeding experiments. This procedure resulted in the synthesis of two polypeptide chains (α and β) of globin.

Further, in a lysate of rabbit reticulocytes, the addition of mammary "messenger RNAs" brought about a significant fall in globin synthesis, but at the same time proteins of higher molecular weight than globin were elaborated. Of these, the one possessing the greatest radioactivity behaved on SDS polyacrylamide gel electrophoresis

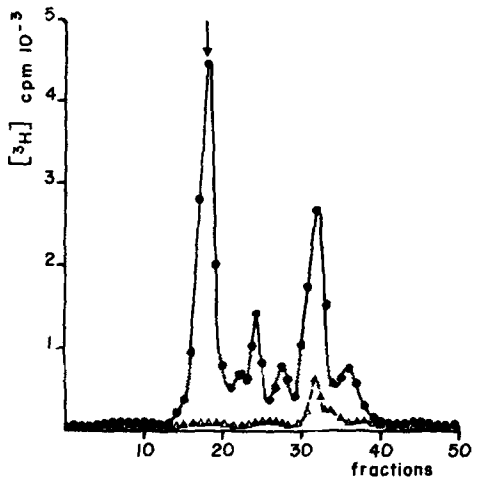

Fig. 6 - Characterization of protein synthesized in a cell free mammary system in response to mammary messenger RNA. The products were analysed by SDS polyacrylamide gel electrophoresis. Migration was from left to right.

Δ——Δ Polypeptide synthesized one to endogenous message

●——● Polypeptide synthesized in the presence of mammary messenger RNA

The arrows indicated the position of α_S casein used in internal marker

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in a manner identical with that of ovine α_S casein (Fig. 7).

The polypeptides which had been synthesized under the influence of bound polyribosomes "messenger RNAs" included one which was precipitable with an antiserum to ovine α_S casein. After dissociation of the antigen-antibody complex by heat and subsequent electrophoresis on polyacrylamide gel containing SDS, there was a single radioactive peak which migrated at a rate identical to marker α_S casein.

III - DISCUSSION

The foregoing results show that it was possible to demonstrate messenger activity in RNA fractions isolated from bound polyribosomes of ovine mammary gland and which sedimented as polydisperse components on sucrose gradient (between 7 S and 17 S). It is not yet possible to state positively if the various molecular forms of RNA, revealed by polyacrylamide gel electrophoresis, corres-

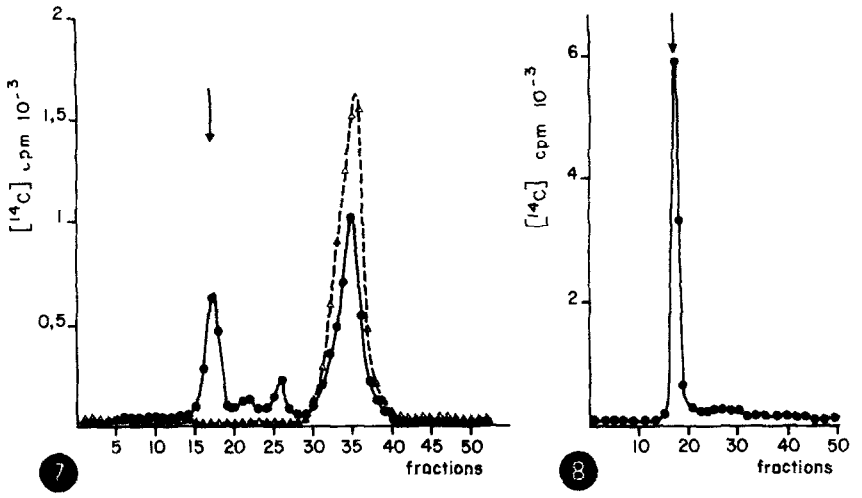

Fig. 7 - Electrophoretic profile of polypeptide synthesized by reticulocyte lysate with (●——●) and without (Δ——Δ) the addition of "mammary messenger RNAs". Duration of incubation : 90 min. with ^{14}C proline and ^{14}C isoleucine. The arrows indicated the position of α_s casein used in internal marker.

Fig. 8 - SDS acrylamide gel electrophoresis of the precipitable radioactivity with an antiserum to ovine α_s casein

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pond to specific coding messengers for the different milk proteins. However, one of these RNAs appeared to be responsible for the synthesis of a milk protein with electrophoretic and immunological properties analogous to α_s casein, the main constituent of ovine milk. A more detailed analysis of the nature of the various messenger RNA fractions is currently being undertaken. The results also show that the translation of mammary messenger RNA for α_s casein does not demand the presence of specific initiation factors. These observations are in agreement with results obtained using other eukaryotic systems (9-21). However, the present experiments constitute the first demonstration of this fact for mammary tissue.

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