High-Molecular-Weight Nuclear Polyadenylate-Containing Ribonucleic Acid isolated from the Lactating Guinea-Pig Mammary Gland contains Milk-Protein Messenger Ribonucleic Acid Sequences

By Ian C. BATHURST, Roger K. CRAIG* and Peter N. CAMPBELL
Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London W1P 7PN, U.K.

(Received 3 May 1979)

1. Steady-state poly(A)-containing nuclear RNA was isolated from the lactating guinea-pig mammary gland and analysed by sucrose-gradient centrifugation under denaturing conditions. 2. Nucleic acid-hybridization studies demonstrated the presence of small amounts of high-molecular-weight RNA species containing milk-protein mRNA sequences sedimenting at 25S. These were not found in the post-nuclear supernatant, where milk-protein sequences sedimented only between 12S and 15S; these were also the predominant species in the nuclear fraction. 3. The results are consistent with the transcription of milk-protein genes as high-molecular-weight precursor RNA species, 3–4 times as large as the active mRNA species isolated from the post-nuclear fraction.

Considerable evidence has recently accumulated which demonstrates that single-copy genes in higher organisms are transcribed as high-molecular-weight precursor molecules. Rapid post-transcriptional processing and modification of these precursors then occurs, resulting in the appearance of lower-molecular-weight cytoplasmic mRNA species (see Ross, 1976; Rabbitts, 1978; Roop et al., 1978; Schibler et al., 1978; Strair et al., 1978).

We wish to investigate the mechanisms involved in the hormonal control of milk-protein gene expression. As a preliminary step towards this objective we have studied the size distribution of steady-state nuclear poly(A)-containing RNA species which contain milk-protein mRNA sequences. During these studies we have identified a population of high-molecular-weight RNA molecules containing milk-protein mRNA sequences with an estimated length of 4100 nucleotides (25S). These have not been found in the post-nuclear fraction, and represent only a small fraction of the milk-protein mRNA sequences located in the nucleus.

Experimental

Materials

[5-3H]dCTP (22 Ci/mol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals, enzymes and solvents were of A.R. grade and obtained from sources previously described (Craig et al., 1976, 1978, 1979). Avian-myeloblastosis-virus reverse transcriptase (lot no. G-678) was provided by Dr. J. W. Beard, Life Sciences Inc., St. Petersburg, FL 33707, U.S.A. Safeguards against RNA degradation by exogenous ribonucleases were as described previously (Craig et al., 1976, 1979), as was the preparation of solutions.

Isolation of poly(A)-containing nuclear RNA

Nuclei were isolated from the lactating guinea-pig mammary gland 3–6 days post partum, essentially as described by Penman (1969), except that the gland was homogenized in the presence of 1% (v/v) Triton X-100 and 6 mM-spermidine. Total nucleic acid was isolated by a combination of proteinase-K digestion followed by phenol/chloroform extraction, and the DNA was digested with ribonuclease-free deoxyribonuclease (Zimmerman & Sandeen, 1966) as described by Kwan et al. (1977). Nuclear poly(A)-containing RNA was then isolated by affinity chromatography on oligo(dT)-cellulose, as was poly(A)-containing RNA from the post-nuclear fraction of the lactating guinea-pig mammary gland (Craig et al., 1976). Typically the glands of one lactating guinea pig (8 g wet wt.) yielded 16–24 μg of poly(A)-containing nuclear RNA (A260 unit ≤ 40 μg).

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

High-specific-radioactivity 3H-labelled cDNA (1.45×10^7 d.p.m./μg) was prepared against post-nuclear poly(A)-containing RNA by using avian-myeloblastosis-virus reverse transcriptase (Craig et al., 1979). The abundant (milk-protein) sequences were then preferentially isolated by selective low-R₀₄₅ hybridization followed by S₁-nuclease digestion of the single-stranded cDNA species (see Craig et al., 1979).

Abbreviation used: cDNA, complementary DNA.

* To whom reprint requests should be addressed.
High-specific-radioactivity $^3$H-labelled DNA complementary to highly purified rabbit globin mRNA was also prepared as described by Craig et al. (1979).

**Size estimation of poly(A)-containing RNA species containing specific mRNA sequences**

The distribution of nuclear and post-nuclear poly(A)-containing RNA species was determined by centrifugation through 12 ml 5–20% (w/v) sucrose gradients in the presence of 75% (v/v) formamide containing 3 mM-Tris/HCl, pH 7.4 at 25°C, and 3 mM-EDTA. RNA samples (8–80 µg/gradient) were freeze-dried, dissolved in 75% (v/v) formamide containing 3 mM-Tris/HCl, pH 7.4 at 25°C, and 3 mM-EDTA, and then heated to 45°C for 15 min (Suzuki et al., 1972) before application to the gradients. These were then centrifuged in the SW 40 rotor of a Beckman L5-65 ultracentrifuge at 198000 gav for 40 h at 25°C, and fractionated as described previously (Craig et al., 1979). *E. coli* tRNA (10 µg) was added to each fraction as a co-precipitant, and the total RNA recovered by precipitation overnight at −20°C in silicone-treated polypropylene centrifuge tubes after the addition of 0.05 vol. of 2 M-NaCl and 3 vol. of ethanol. Precipitates were sedimented by centrifugation at −5°C in the SW 56 rotor of a Beckman L5-65 ultracentrifuge for 30 min at 100000 gav. The sedimented RNA was washed once in ethanol, resedimented, freeze-dried, and finally dissolved in Chelex-treated double-distilled water (10 µl). To identify the presence of specific mRNA sequences, 2 µl of each fraction was removed, freeze-dried in presence of 700–800 c.p.m. of the appropriate $^3$H-labelled cDNA species, and then redissolved in 2 µl of 0.24 M-sodium phosphate, pH 6.9, containing 1 mM-EDTA and 0.2% (w/v) sodium dodecyl sulphate. Hybridizations were performed for 30 h at 70°C, and the amount of $^3$H-labelled cDNA hybridized was determined by S1-nuclease digestion as described previously (Craig et al., 1979).

28S and 18S rRNA species used as molecular-weight marker species were isolated from rabbit reticulocytes (Craig et al., 1976) and their relative positions on the gradients monitored at 270 nm.

**Results and Discussion**

Comparison of the size distribution of milk-protein mRNA sequences in both the nuclear and the post-nuclear poly(A)-containing RNA populations isolated from the lactating guinea-pig mammary gland (Fig. 1a) by using the abundant $^3$H-labelled cDNA population as the hybridization probe demonstrated that the vast majority (over 90%) of the hybridized sequences in both populations co-sedimented as a single broad peak, of average sedimentation coefficient 14.6 S. However, whereas essentially none of the input cDNA probe hybridized to post-nuclear poly(A)-containing RNA species

which sedimented at a greater rate than an 18S rRNA marker, low but significant amounts of the input cDNA were protected from S1-nuclease digestion by nuclear poly(A)-containing RNA species sedimenting
faster than the 18S marker species. To ascertain whether these represented high-molecular-weight RNA species which contained milk-protein mRNA sequences, as opposed to non-specific hybridization, all fractions containing nuclear RNA species greater than 18S were pooled, and their size distribution was reassessed by sucrose gradient centrifugation in the presence of formamide. As a control, all post-nuclear poly(A)-containing RNA species sedimenting faster than 18S were also re-analysed.

The results (Fig. 1b) demonstrate that, although some hybridizable milk-protein mRNA sequences were present in the post-nuclear fraction, these sedimented only at the expected position of the milk-protein mRNA species. No higher-molecular-weight post-nuclear poly(A)-containing RNA species containing milk-protein mRNA sequences sedimenting faster than the milk-protein mRNA species could be detected. In sharp contrast, re-analysis of the nuclear poly(A)-containing RNA revealed two populations of RNA species possessing milk-protein mRNA sequences. One co-sedimented with the post-nuclear population, and the other as a distinctive peak with an average sedimentation coefficient of 25S.

Although this result is consistent with the presence in the nucleus of small amounts of high-molecular-weight poly(A)-containing RNA which possess the milk-protein mRNA sequences, it is of importance to consider both the purity of the cDNA hybridization probe (see Haynes et al., 1978) and also aggregation phenomena (see Macnaughton et al., 1974). The problem of contaminating cDNA species may be excluded, as maximum hybridization of the putative precursor species (fraction 9, Fig. 1b) represents over 75% protection of the input abundant cDNA probe, suggesting in turn that most if not all of the milk-protein mRNA sequences may be found in high-molecular-weight nuclear poly(A)-containing RNA species.

In order to consider aggregation phenomena, purified rabbit globin mRNA was added to nuclei isolated from the lactating guinea-pig mammary gland immediately before proteinase-K digestion. Poly(A)-containing globin sequences were then copurified with the poly(A)-containing nuclear RNA species, and the relative distributions of globin and milk-protein mRNA sequences determined on the same denaturing gradient. The results (Fig. 2a) reveal that the vast majority of both the globin and milk-protein mRNA sequences sediment at the expected positions on the gradient (10S and 14.6S respectively), although in this experiment some hybridizable material representing both globin and milk-protein mRNA sequences was observed at the bottom of the gradient, suggesting either that some aggregation had occurred, or alternatively that the RNA was not completely in solution before centrifugation proceeded. However, re-analysis of the RNA sedimenting faster than 18S (Fig. 2b) confirmed our previous observation (Fig. 1b) that a high-molecular-weight poly(A)-containing RNA species of nuclear location which contained milk-protein mRNA sequences could be found in the lactating guinea-pig mammary gland. Moreover, it seemed likely that this was not due to aggregation, as globin mRNA sequences co-

![Fig. 2. Detection of a 25 S nuclear poly(A)-containing RNA species containing milk-protein mRNA sequences when isolated under conditions that prevented aggregation of rabbit globin mRNA](image-url)
purified under identical conditions could only be detected in a position corresponding to the processed cytoplasmic 10S species.

Further studies using nuclear poly(A)-containing RNA treated with either glyoxal (McMaster & Carmichael, 1977) or 85% formamide at 45°C for 15 min, followed by gradient analysis, all resulted in the identification of the 25S nuclear species (I. C. Bathurst & R. K. Craig, unpublished work), thereby substantiating our conclusions detailed above.

The identification of a steady-state presumptive high-molecular-weight nuclear RNA precursor for cytoplasmic milk-protein mRNA species is very similar to recent studies, utilizing either purified cDNA or cloned sequences as hybridization probes, which demonstrate the presence in nuclei of cells and tissues of high-molecular-weight RNA species which contain mRNA sequences coding for albumin (Strair et al., 1978), the heavy- and light-chain immunoglobins (Rabbitts, 1978; Schibler et al., 1978), globin (Macnaughton et al., 1974) and ovalbumin (Roop et al., 1978). In common with these reports, the steady-state poly(A)-containing nuclear RNA species which contain the milk-protein mRNA sequences appear to be 3–4 times as long [4100 nucleotides; calculated by the formulae of Spirin (1963) directly from the sedimentation coefficient] as the active mRNA species isolated from the post-nuclear fraction (1350 nucleotides; see Craig et al., 1979). Thus it seems likely that the milk-protein genes of the lactating guinea-pig mammary gland are transcribed as high-molecular-weight transient precursor species, and that these are subsequently processed and finally transported into the cytoplasm as functional mRNA. Whether the 25S nuclear poly(A)-containing RNA species represent the primary milk-protein transcripts or an intermediate in the post-transcriptional mechanisms has yet to be established.

We thank the Wellcome Trust for supporting this work and Dr. J. Beard and Dr. J. Gruber for the gift of avian-myeloblastosis-virus reverse transcriptase.

References