Isolation and Characterization of Purified Rat Casein Messenger Ribonucleic Acids†

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ABSTRACT: Purification of casein messenger ribonucleic acids (mRNAs) from lactating rat mammary gland RNA has been accomplished by a combination of sizing techniques, including Sepharose 4B chromatography and preparative agarose–urea gel electrophoresis, and affinity chromatography of poly(adenylic acid)-containing mRNA on oligo(dT)-cellulose. The separation of the individual casein mRNAs into discrete molecular species free of apparent ribosomal RNA contaminants was facilitated by the use of denaturing conditions either prior to or during each of the fractionation procedures. Two casein mRNA fractions were isolated: (1) a 15S mRNA doublet which directed the synthesis of the two largest rat caseins in the wheat-germ, cell-free, translation assay, and (2) a 12S mRNA which migrated as a single species during agarose–urea gel electrophoresis and directed the in vitro synthesis of the smallest of three rat caseins. These mRNAs had apparent molecular weights of 450 000 ± 30 000 and 320 000 ± 25 000 and contained poly(adenylic acid) sequences at their 3′ termini ranging from 15 to 150 residues with number average lengths of 42 and 38 adenosines, respectively. The purity of the isolated casein mRNAs was determined both by agarose–urea gel electrophoresis and by a careful comparison of the total products synthesized in the wheat-germ translation assay with those recognized by a specific casein antibody using an indirect immunoprecipitation technique. The specificity of the indirect immunoassay procedure was demonstrated by the selective displacement by purified rat casein of greater than 95% of the radioactive product synthesized in the cell-free system. Under optimal translation conditions for casein mRNA, at least 90% of the released protein synthesized in response to the 15S casein mRNA was specifically immunoprecipitable, representing a 178-fold purification compared with the initial RNA extract. Using these techniques a comparable purification was also obtained for a 15S mouse casein mRNA fraction. Finally, an analysis by fluorography on 5–20% (w/v) polyacrylamide gradient slab gels of the total proteins synthesized in response to both the 15S and 12S casein mRNAs revealed a close correspondence with those proteins which were specifically immunoprecipitated.

Previous studies from numerous laboratories (Williamson et al., 1971; Suzuki and Brown, 1972; Favre et al., 1974; Stavnezer et al., 1974; Gielen et al., 1974; Haines et al., 1974; Mandal et al., 1974) including our own (Rosen et al., 1974, 1975a; Woo et al., 1975) have demonstrated that specific mRNAs[1] may be purified from total cell or polysomal RNA extracts. Purification usually requires the combination of conventional RNA sizing techniques with affinity chromatography of the poly(A)-containing mRNA on either oligo(dT)-cellulose (Aviv and Leder, 1972) or poly(U)-Sepharose (Gaye and Houdebine, 1975). Furthermore, during each of the procedures employed it is essential to prevent mRNA aggregation in order to allow for the isolation of discrete mRNA species (Haines et al., 1974; Gielen et al., 1974; Rosen et al., 1976). Messenger RNA purity may be ascertained by a careful product analysis using a cell-free translation system with a low level of endogenous protein synthesis (Rosen et al., 1975a). However, an additional independent criterion such as gel electrophoresis of the isolated mRNA under denaturing conditions should also be employed (Morrison et al., 1974; Lizardi et al., 1975; Rosen et al., 1975a; Spohr et al., 1976).

Identification of three different ewe casein mRNA activities in a 20-fold purified poly(A)-containing RNA extract has been previously reported (Gaye and Houdebine, 1975). A recent report has suggested that at least two of these ewe casein mRNAs can be partially resolved from each other by the immunoprecipitation of polysomes containing the nascent chains for these caseins (Houdebine and Gaye, 1976). However, only small quantities of these individual casein mRNAs could be isolated by this procedure limiting their further characterization. In addition, no attempt was made to characterize the total products specified by these isolated mRNAs in a cell-free translation assay making an estimation of their absolute purities difficult. Furthermore, application of this immunoprecipitation technique to the rat mammary gland has proven not to be feasible due to the difficulties encountered in isolating sufficient amounts of undegraded polysomes and because of the weak antigenicity of the rat casein (Rosen, unpublished observations).

Previous studies from our laboratory revealed that casein mRNA activity comprises approximately 50% of the total mRNA activity in RNA extracts isolated from lactating rat mammary glands (Rosen et al., 1975b). This facilitated both the initial purification of casein mRNA and the partial resolution of two rat casein mRNA fractions. In this paper the isolation of sufficient amounts of these purified rat casein mRNAs to allow their preliminary characterization is reported. Several problems were inherent in the isolation and characterization of the rat casein mRNAs: (1) in an analogous fashion to the hemoglobin mRNAs (Williamson et al., 1971; Gielen et al., 1974; Morrison et al., 1974), the removal of

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[1] Abbreviations used: Na_2EDTA, disodium ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; 12 S and 15 S were used to designate the relative positions of the casein mRNAs during gel electrophoresis and were not $s_{20,w}$ determined by centrifugation; poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); dT, deoxythymidine; mRNA and rRNA, messenger and ribosomal ribonucleic acids, respectively; cDNA, complementary deoxyribonucleic acid; Tris, tris(hydroxymethyl)aminomethane.

noncasein mRNA activities and rRNA has been considerably easier than the complete resolution of the individual casein mRNA species; (2) furthermore, since caseins are known to be both phosphoproteins and glycoproteins (Taborsky, 1974), the proteins synthesized in the cell-free translation assay could not be directly compared with the native rat caseins; and (3) limited sequence homology within a single casein species and between the individual caseins presented a further complication (Taborsky, 1974). Despite these difficulties, the individual rat casein mRNAs have been partially resolved and used to generate individual cDNA hybridization probes (Rosen and Barker, 1976). The preliminary analysis of the products directed by these isolated casein mRNAs in the wheat-germ translation assay is also presented.

Experimental Procedures

Casein mRNA Isolation and Purification. RNA was isolated from rapidly frozen 5 to 10 day lactating rat mammary glands by a phenol–chloroform–sodium dodecyl sulfate extraction procedure at pH 8.0 (Rosen et al., 1975a,b). The methods utilized for the chromatography of the lactating RNA extract on either oligo(dT)-cellulose or Sepharose 4B have been described in detail elsewhere (Rosen et al., 1975a,b, 1976) with the following modifications. The RNA prior to each of these chromatographic procedures was dissolved in a low ionic strength buffer containing either 0.01 M Tris-HCl, pH 7.6, 0.001 M Na_2EDTA for the dT-cellulose chromatography or 0.001 M Na_2EDTA, pH 5.0, alone for the Sepharose 4B chromatography. RNA aggregates were denatured by heating at 70 °C for 1 min, followed by rapid cooling at 4 °C. RNA samples were either directly applied to the Sepharose 4B column equilibrated in a buffer containing 0.1 M $NaC_2H_3O_2$–0.001 M Na_2EDTA, pH 5.0, at 4 °C or KCl was added to a final concentration of 0.5 M and dT-cellulose affinity chromatography was performed as previously reported (Rosen et al., 1975a). This technique not only improved the resolution of individual mRNAs during Sepharose 4B chromatography, but also increased both the recovery and resolution of poly(A)-containing mRNA from rRNA during dT-cellulose chromatography. Both analytical and preparative agarose–urea gel electrophoresis were performed on 3% agarose gels (Bio-Rad) in 6 M urea and 0.025 M citric acid, pH 3.5 (Rosen et al., 1975a; Woo et al., 1975). Gels were stained for 30 min in a 1% methylene blue–15% acetic acid solution, destained overnight in water, and scanned at 600 nm using a Gilford 2400S spectrophotometer.

Product Analysis in the Wheat-Germ Translation Assay. The preparation of the wheat-germ S-30 and the conditions of the assay were essentially similar to those previously described (Roberts et al., 1975). However, the assay was optimized for the translation of casein mRNA as follows: (1) the KCl concentration was increased to 104 mM (see Results); (2) the creatine kinase concentration was decreased to 10 μg/ml; (3) 40 μM spermine (Sigma) was added; and (4) the pH was lowered from 7.6 to 7.0. Assays (150 μl) were routinely employed containing L-[5-^{3}H]proline (Schwarz/Mann, 43 Ci/mmol) at a final concentration of 2 μM and the incubations allowed to proceed for 2 h at 25 °C. Unless otherwise specified, the ribosomes were then removed by centrifugation at 105 000g for 1 h at 4 °C in Beckman type 75 Ti rotor. Total mRNA and casein mRNA activities were determined by measuring the incorporation of [^{3}H]proline into released polypeptides using Cl_3COOH precipitation or immunoprecipitation, respectively. In some instances the ribosomal pellets were dissolved in 100 μl of the standard antibody buffer containing 2% Triton X-100–0.01 M proline–0.01 M sodium phosphate–0.14 M NaCl (pH 7.5). Total mRNA activity was then determined using 20 μl aliquots of either the total incubation mixture, the postribosomal supernatant, or the ribosomal pellet as previously described (Rosen et al., 1975a).

An indirect immunoprecipitation assay was utilized to measure casein mRNA activity using a rabbit IgG fraction prepared against a mixture of the three purified rat caseins (Rosen et al., 1975b). The anticasein IgG fraction (2 μl) was incubated with a 100-μl aliquot of the cell-free product in the standard antibody buffer for 30 min at 25 °C followed by an overnight incubation at 4 °C. Thirty microliters of a goat anti-rabbit IgG serum (Miles-Yeda) was then added and a precipitation complex was formed by incubating the mixture at 25 °C for 30 min followed by at least a 3-h incubation at 4 °C. The mixtures were then layered over a 1-ml cushion of 1 M sucrose prepared in the antibody buffer and the specific immunoprecipitate was isolated and quantitated as previously described (Rosen et al., 1975b). The specificity of this procedure was determined by measuring the ability of various proteins to compete for antibody binding sites with the newly synthesized radioactive caseins.

Product analysis of both the total peptides synthesized in the wheat germ system as well as those recognized by the specific antiserum was performed on either a 25-cm, 5–20% (w/v), or a 10-cm, 5–15% (w/v) polyacrylamide gradient slab gel using the buffer system of Laemmli (1970). The tritiated polypeptides were detected by fluorography by the method of Bonner and Lasky (1974). The relative amounts of each of the products synthesized were quantitated using a Quick Scan autoradiogram scanning device (Helena Laboratories).

Characterization of Casein mRNA. The molecular weights of the isolated casein mRNAs were determined by two independent methods. In the first method of the migration of the purified casein mRNAs on either 3% agarose–urea gels or 4% polacrylamide slab gels containing 99% formamide (Boedtker et al., 1973) were compared with other mRNA standards of known molecular weights: MS-2 RNA was obtained from Miles Laboratories, partially purified hemoglobin mRNA was prepared from a reticulocyte lysate by the method of Gielen et al. (1974), and purified ovalbumin mRNA was isolated as previously described (Woo et al., 1975).

In the second method the molecular weights were determined by a chemical method in which both the length of the poly(A) segment and the percentage of the RNA that is poly(A) by hybridization with [^{3}H]poly(U) were determined (Woo et al., 1975). Localization of the poly(A) sequence at the 3′ termini of casein mRNA and the determination of its length were accomplished essentially by the method of Burr and Lingrel (1971) as modified by Monahan et al. (1976). Briefly, this procedure entailed the labeling of the 3′-terminal adenosine with [^{3}H]$NaBH_4$ after periodate oxidation, followed by digestion of the RNA with pancreatic and T_1 RNases, and finally electrophoresis of the resistant poly(A) fragment on 12.5% polyacrylamide gels in a Tris-citric acid buffer (pH 8.0). Poly(A) standards of known lengths were obtained from Miles Laboratories, labeled in a comparable fashion and used as markers during gel electrophoresis. The percentage of the RNA that is poly(A) was determined by hybridization with [^{3}H]poly(U) (Schwarz/Mann, 23.6 mCi/mmol) using a poly(A) standard curve (Rosen et al., 1974).

Results

We have previously reported the partial purification of casein mRNA from other mRNAs and most of the 18S rRNA

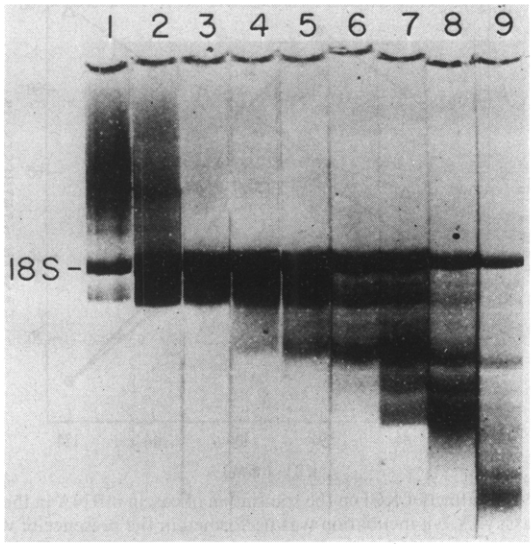

FIGURE 1: Chromatography of dT-bound poly(A)-containing RNA on Sepharose 4B. Oligo(dT)-cellulose-bound, lactating RNA (18.6 mg), dissolved in 0.001 M Na_2EDTA, pH 5.0, at a concentration of 1.5 mg/ml, was heated at 70 °C for 1 min and rapidly cooled to 4 °C. Chromatography was performed on a 2.6 × 90 cm Sepharose 4B column equilibrated with 0.1 M sodium acetate, 0.001 M Na_2EDTA, pH 5.0, at a flow rate of 10 ml/h. Nine-milliliter fractions were collected and the RNA was recovered by ethanol precipitation. Analysis of the following fractions was performed on 3% agarose–urea gels as described under Experimental Procedures: gel 1, 15 μg of RNA, fraction 24; gel 2, 21 μg of RNA, fraction 26; gel 3, 14 μg of RNA, fraction 28; gel 4, 17 μg of RNA, fraction 29; gel 5, 13 μg of RNA, fraction 31; gel 6, 19 μg of RNA, fraction 32; gel 7, 17 μg of RNA, fraction 34; gel 8, 16 μg of RNA, fraction 36; gel 9, 12 μg of RNA, fraction 40.

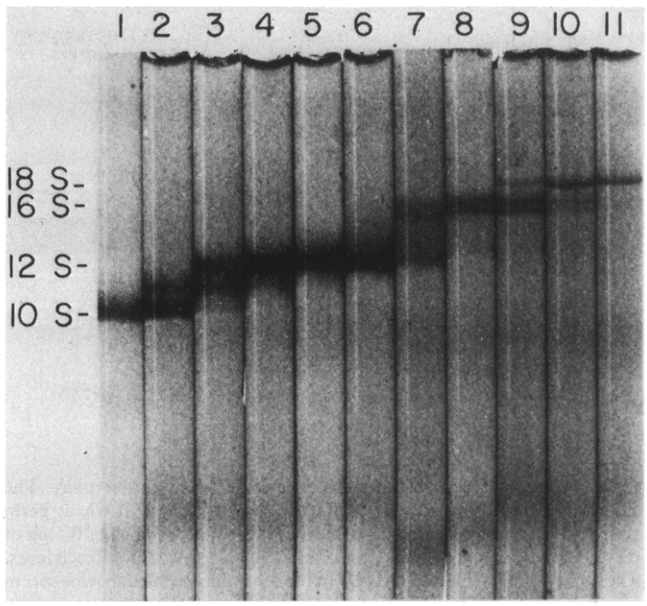

FIGURE 2: Preparative agarose gel electrophoresis of Sepharose 4B fraction II after rechromatography on dT-cellulose. A partially purified 12S casein mRNA fraction (1.3 mg, re-dT-cellulose bound II) was electrophoresed on a 40-ml, 3% preparative agarose–urea gel for 24 h at 30 mA at 4 °C. RNA was eluted at flow rate 24 ml/h and 8-ml fractions were collected. Following electrophoresis the RNA was recovered by ethanol precipitation after adjusting the Na^+ concentration to 0.5 M, and the RNA pellets were resuspended in 5 ml of 70% ethanol at 4 °C and finally dissolved in 0.25 ml of H_2O. Then 30 μl of each fraction was analyzed on analytical 3% agarose–urea disc gels as described in the Experimental Procedures: gel 1, fraction 35; 2, 38; 3, 41; 4, 43; 5, 45; 6, 48; 7, 51; 8, 54; 9, 58; 10, 61; 11, 65.

(Rosen et al., 1975b). In the original procedure no attempt was made to resolve the individual casein mRNAs, and 18S rRNA was present in the final preparation. In addition, the direct immunoprecipitation assay did not permit the quantitative assessment of the percentage of casein mRNA activity without the use of ^{125}I-labeled internal casein standards. Finally, the wheat-germ translation assay was not optimized for casein mRNA translation.

In order to resolve partially the individual casein mRNAs two modifications of the original procedure were employed. Sepharose 4B chromatography was performed on a heat-denatured, poly(A)-enriched, lactating mRNA preparation isolated by previous dT-cellulose chromatography. The total casein mRNA activity was distributed across the 18S rRNA peak (Rosen et al., 1975b) which still comprises the majority of the RNA mass in the dT-bound RNA preparation. Analysis of each of the fractions across the 18S rRNA peak by electrophoresis on 3% agarose–urea gels revealed a distribution of RNAs smaller than 18 S (Figure 1). Specifically, an intense RNA doublet at approximately 15 S (gels 2–4) and a second species of RNA at 12 S (gels 5–8) were observed. These RNAs have previously been shown to contain casein mRNA activity by elution from the agarose gels and translation in the wheat-germ assay (Rosen et al., 1975b). In addition to the 15S and 12S casein mRNAs, the latter fractions also contained a series of smaller RNAs of 9 to 12 S in size. Thus, by careful fractionation of heat-denatured, poly(A)-enriched RNA, the partial resolution of two of the casein mRNA fractions from each other and some presumptive smaller mRNAs was accomplished. Moreover, this technique removed any contaminating 28S rRNA, which was adsorbed to the agarose under the ionic conditions employed (Rosen et al., 1975a). The majority of the DNA and the smaller 4 and 5S RNAs had been previously removed by dT-cellulose chromatography.

The majority of the remaining 18S rRNA was next removed from each of the Sepharose 4B casein mRNA fractions by rechromatography of these heat denatured mRNAs on dT-cellulose. A final and even more precise sizing technique, preparative agarose–urea electrophoresis, was then utilized to resolve the casein mRNA fractions from each other and any other potential contaminants. An example of the efficacy of the 3% agarose preparative gel for the fractionation of a rather small size distribution of RNAs (10 to 18S) into discrete species is shown in Figure 2. The 12S casein mRNA was completely separated from the 15S casein mRNA doublet, the small amount of remaining 18S rRNA, and several smaller RNA species.

Before determining the extent of purification of each of the casein mRNAs in the wheat-germ translation assay, the specificity of the indirect immunoprecipitation assay was studied. The ability of purified rat casein, rat α-lactalbumin, and bovine serum albumin to displace radioactive casein synthesized in vitro was explored (Figure 3). Approximately 50% of the total protein synthesized in vitro in response to a dT-cellulose-bound lactating RNA preparation was specifically precipitated by the addition of the rabbit anticasein IgG and goat anti-rabbit IgG. Greater than 95% of the radioactivity could be displaced by the addition of 10 μg of purified rat casein, but no displacement was observed with either 10 μg of purified rat α-lactalbumin or 100 μg of bovine serum albumin. No cross-reactivity was detected when 10 μg of bovine α_s casein was used as the competitor (data not shown). A 2.6% level of nonspecific trapping was accounted for by the inability of the

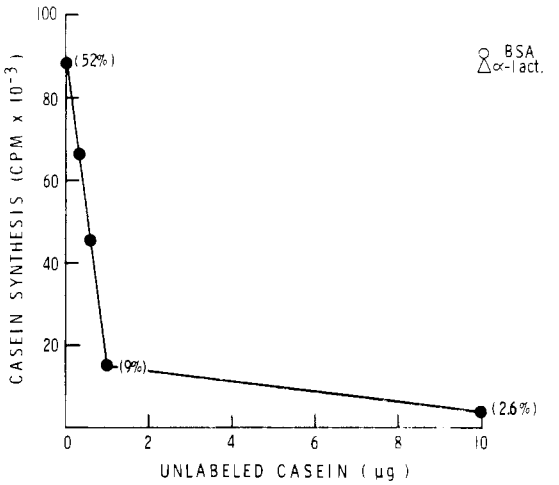

FIGURE 3: Specificity of the indirect immunoprecipitation assay. The immunoprecipitation reaction was performed on the total wheat germ reaction mixture following a 2-h incubation in the presence of 0.5 μg of dT-bound, lactating RNA as described in the Experimental Procedures. The ability of unlabeled casein (●) in the amounts specified, bovine serum albumin, 100 μg (○), or purified rat α-lactalbumin, 10 μg (△), to compete with the binding of the [^{3}H]casein to a rabbit anticasein IgG was determined. Purified rat α-lactalbumin was kindly supplied by Dr. Kurt Ebner.

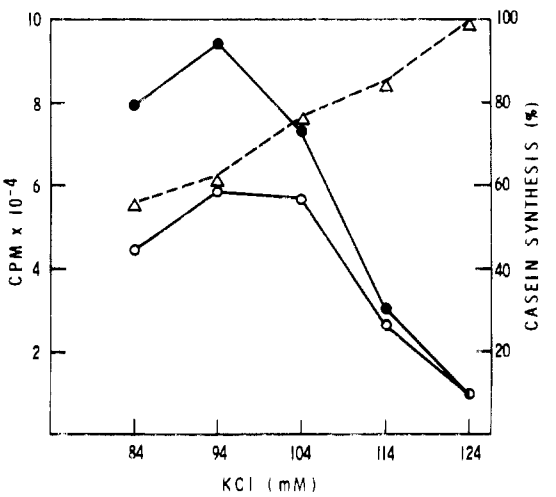

FIGURE 4: Effect of KCl on the translation of casein mRNA in the wheat germ assay. A 2-h incubation was performed in the presence of varying concentrations of KCl with all other components present at the concentrations specified in Experimental Procedures. Both the total protein synthesis (●) and specific casein synthesis (○) were determined in response to the addition of 0.14 μg of a purified 15S casein mRNA fraction. These assays were performed on the total wheat germ assay mixture. The percentage of casein synthesis is shown by △- - -△.

unlabeled casein to displace totally the cell-free product and was characteristic of the immunoprecipitation assay performed on the total reaction mixture.

It was necessary to optimize the wheat-germ assay system for the translation of casein mRNA prior to any further product analysis. Several recent reports have suggested that the ionic conditions in the wheat-germ cell-free system can markedly affect both chain completion (Schmeckpeper et al., 1974) and the optimal translation of different mRNAs (Vermorken et al., 1975; Benveniste et al., 1976). Thus, the effect of varying the KCl concentration on the synthesis of casein in vitro was studied (Figure 4). The assay of both casein and total mRNA activities using the purified 15S casein mRNA was performed on the total wheat-germ reaction products and not on the released polypeptides alone. Under these conditions, casein mRNA activity increased from approximately 60 to 100% of the total activity when the KCl concentration increased from 84 to 124 mM. Optimal incorporation of the [^{3}H]proline into total Cl_3CCOOH-precipitable protein occurred at 94 mM KCl, while casein mRNA activity (immunoprecipitable product) plateaued at 94–104 mM KCl. This effect of varying the monovalent cation appeared to be primarily on the elongation and release of immunoreactive casein since greater than 90% of the total mRNA activity was found to be casein mRNA activity at 104 mM when the assay was performed on the released polypeptides (see Table I). Furthermore, the relative amounts of the three different caseins synthesized in the cell-free system were unaffected by raising the [K] from 84 to 124 mM (data not shown). However, at the higher [K] the initiation of both total protein and casein synthesis was markedly inhibited (five- to tenfold). Therefore, 104 mM was chosen as the optimal KCl concentration and both the Cl_3CCOOH and immunoprecipitation assays were performed on the released polypeptides. Finally, a careful analysis of such other variables as the Mg^{2+} concentration, pH, and the addition of spermine or creatine kinase demonstrated that these factors could stimulate the total protein synthetic capacity of the wheat-germ assay (approximately twofold), but did not affect the ratio of casein to total protein synthesis (data not shown).

Each of the RNA fractions obtained at the different stages of the purification scheme was next assayed using these optimized translation conditions (Table I). Several concentrations of each RNA sample were utilized and the specific activities of the mRNA fractions determined from the linear portions of the assay. The percent release of both total protein and immunoreactive casein was determined. Starting with 850 mg of total lactating RNA, approximately 200 μg of the purified 15S casein mRNA and 100 μg of the purified 12S mRNA were obtained. The 15S casein mRNA fraction represented at least 90% casein mRNA activity and was 178-fold purified compared with the initial RNA extract. The efficiency of chain release in the cell-free system was also quite good using the purified 15S casein mRNA, i.e., almost 80%, and was increased compared with the total RNA (approximately 50%). No marked differences in the release of total peptides or immunoreactive casein were observed, except for the translation of dT-bound RNA where the release of casein was slightly enhanced.

In contrast to the purified 15S casein mRNA only 60% of the products specified by the purified 12S casein mRNA fraction were specifically precipitated by the combined anticasein IgG. Although the final total mRNA activities of both the 15S and 12S mRNA fractions were comparable, the 12S casein mRNA displayed only a 95-fold enrichment in casein mRNA activity as compared with the initial RNA sample (Table I). However, this may partially reflect the inability of the anticasein IgG fraction to quantitatively precipitate the smallest in vitro synthesized rat casein (see Discussion and Figure 5).

Using this procedure, the overall recovery of casein mRNA activity (re-dT I + II) was greater than 50% of the initial activity until the final resolution of the 15 and 12S casein mRNAs by preparative gel electrophoresis (Table I). A substantial improvement in both total recovery and resolution of the individual mRNAs was obtained by heat denaturation prior to each of the chromatographic procedures, especially prior to chromatography on dT-cellulose. The final yields of

TABLE I: Purification of Rat Casein mRNA.

Sample	RNA (mg)	Total mRNA Act.[c]	Casein mRNA Act.[c]	$mRNA_C/mRNA_T$ (%)[b]	Fold	% Release: Total mRNA	% Release: Casein mRNA
Total RNA	850[a]	16	8	50		56	49
dT-bound	18.6	469	310	66	37	55	71
Sepharose peak I	4.9	831	640	77	76	78	79
Re-dT bound I	2.0	1630	1350	83	162	69	73
Prep gel 15 S	0.2	1660	1490	90	178	68	78
Sepharose peak II	3.4	734	338	46	40	50	55
Re-dT bound II	1.3	1490	788	53	94	56	58
Prep gel 12 S	0.1	1330	796	60	95	50	54

[a] Isolated from 170 g of pooled, lactating rat mammary glands. [b] C, casein; T, total. A 3% trapping value obtained using an ovalbumin mRNA control was subtracted from each casein mRNA value. [c] Activity in cpm/μg $\times 10^{-3}$.

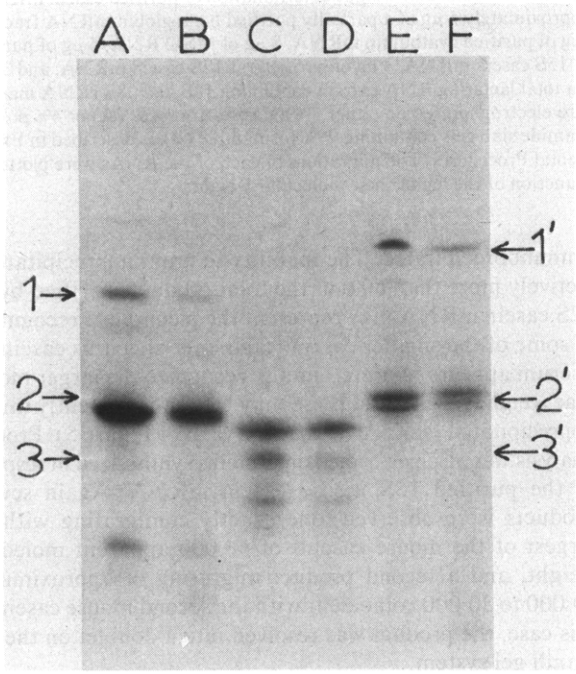

FIGURE 5: Product analysis of the in vitro proteins synthesized in response to purified rat and mouse casein mRNAs. Following a 2-h incubation, both the released total proteins and casein synthesized in the wheat germ assay were assayed as described in Experimental Procedures. Approximately 120 000–200 000 cpm of the total products (slots B, D, and F) and specifically immunoprecipitated caseins (slots A, C, and E) were electrophoresed on a 25-cm, 5–20% polyacrylamide slab gel using the sodium dodecyl sulfate–Tris–glycine system of Laemmli (1970). Electrophoresis was performed at 8 mA for 4 h and then at 15 mA for an addition 13 h. Following electrophoresis the casein standards were localized by staining with Coomassie brilliant blue (0.1% in methanol–acetic acid–H_2O 4:1:5) and fluorography performed for 24 h as described (Bonner and Laskey, 1974). Each 200 μl of wheat germ reaction mixture contained the following amounts of the purified casein mRNA fractions: slots A and B, 0.5 μg of purified rat 15S casein mRNA; slots C and D, 0.43 μg of purified rat 12S casein mRNA; slots E and F, 0.7 μg of purified mouse 15S casein mRNA. The rat casein standards are designated: 1, 42 000; 2, 29 500; 3, 24 500. The mouse casein standards are designated: 1′, 46 000; 2′, 29 500; 3′, 26 500.

the purified 15S and 12S casein mRNAs represented approximately 5% of the initial casein mRNA activity (Table I). The loss in recovery of casein mRNA activity during preparative gel electrophoresis reflected the selection of those fractions containing no cross-contamination with either 18S rRNA

TABLE II: Purification of Mouse Casein mRNA.

Sample	Total mRNA Act.[a]	Casein mRNA Act.[a]	$mRNA_C/mRNA_T$ (%)[b]	Fold
Total RNA	55	21	38	
dT-bound	512	276	54	13
Sepharose peak I	1280	1020	80	49
Re-dT bound	3010	2470	82	119
Sucrose gradient 15 S	3320	3090	93	148

[a] Activity in cpm/μg $\times 10^{-3}$. [b] C, casein; T, total.

or mRNAs <12S, in addition to the resolution of the individual casein mRNAs. However, even when the total casein mRNA activity in all fractions was considered, there was a 50% loss during preparative gel electrophoresis of mRNA activity. This may be due to both partial degradation of mRNA during prolonged electrophoresis and the inability to recover small amounts of mRNA in the dilute eluates by ethanol precipitation.

The general applicability of this purification procedure to the isolation of purified casein mRNAs from other species is demonstrated in Table II. In this case, a comparable purification (148-fold and 90% casein mRNA activity) was obtained for a 15S mouse casein mRNA fraction. Sucrose gradient centrifugation following heat denaturation (Rosen et al., 1975b) was used as the final fractionation procedure instead of preparative agarose electrophoresis. Since only 200 mg of total mouse lactating RNA was used initially, the amount of mouse casein mRNA remaining after rechromatography on dT-cellulose was not sufficient to permit adequate recoveries on the large preparative agarose gel.

In order to further characterize the biological activity of the isolated rat and mouse casein mRNAs a careful product analysis of both the total proteins synthesized in the wheat germ system and those recognized by the specific antisera was performed. Initial experiments utilized 10-cm long, 10% polyacrylamide slab gels employing the $NaDodSO_4$–phosphate system of Weber and Osborn (1969). Although this method allowed a comparison of the total and immunoreactive products, it did not permit adequate resolution of the smaller caseins of 25 000 to 30 000 molecular weight synthesized in vitro (data not shown). Using a 25-cm long, 5–20% (w/v) polyacrylamide

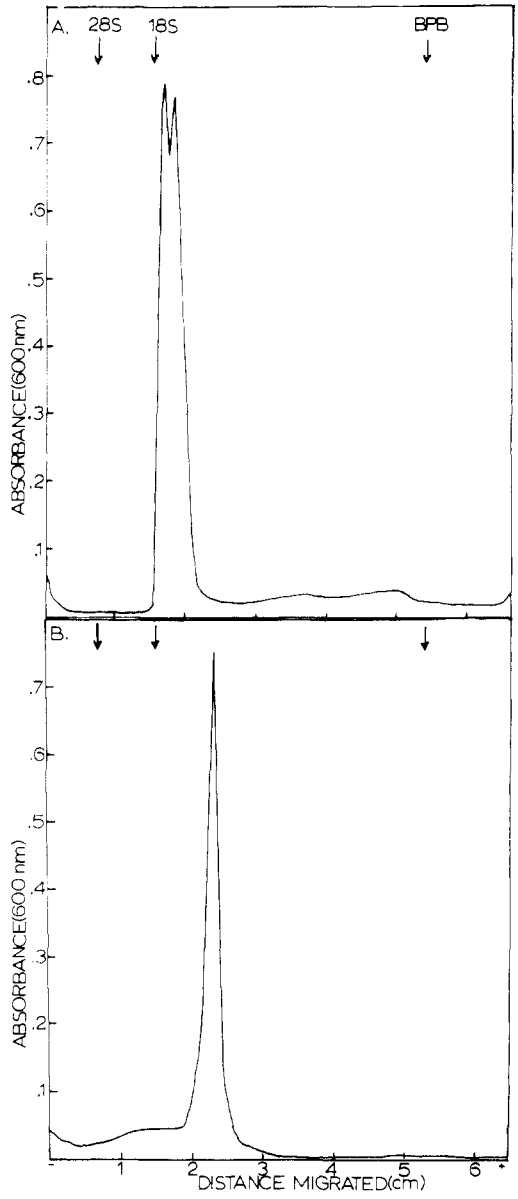

FIGURE 6: Characterization of purified rat casein mRNAs by electrophoresis on 3% agarose gels containing 6 M urea and 0.025 M sodium citrate, pH 3.5, for 6 h at 4 °C. Following staining with 1% methylene blue in 15% acetic acid, the gels were destained and scanned using a Gilford 2400S spectrophotometer containing a 2410 linear transport. The arrows designate the positions of rat 28S and 18S rRNA standards run on a parallel gel and the position of the bromophenol blue dye marker (BPB).

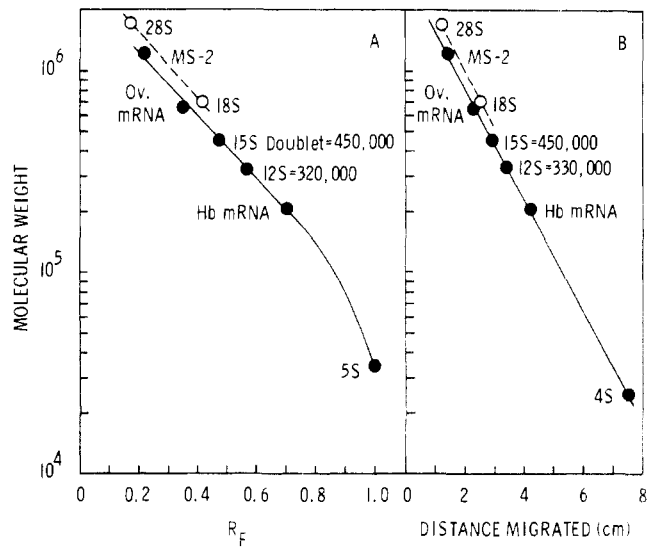

FIGURE 7: Molecular weight determination of purified casein mRNAs. Approximately 5 μg of a partially purified hemoglobin mRNA fraction, 9 μg of purified ovalbumin mRNA, 8 μg of MS-2 RNA, 5 μg of purified rat 15S casein mRNA, 4 μg of purified rat 12S casein mRNA, and 30 μg of a total lactating RNA extract containing 18S and 28S rRNA markers were electrophoresed on either 3% agarose-urea gels (A) or 4% polyacrylamide slab gels containing 99% formamide (B) as described in Experimental Procedures. The migrations of each of the RNAs were plotted as a function of the log of their molecular weight.

gradient slab gel and the stacking $NaDodSO_4$-Tris-glycine system developed by Laemmli (1970), a more accurate comparison of the in vitro products was possible (Figure 5). The 15S rat casein mRNA doublet directed the synthesis of two of the rat caseins (Figure 5A,B): the predominant product of the in vitro translation (>90%) migrated slightly faster than the 30 000 molecular weight casein standard, while the remaining product (<10%) comigrated with the 42 000 molecular weight rat casein. The major protein synthesized in response to the 12S rat casein mRNA migrated more slowly than the smallest rat casein of 25 000 apparent molecular weight (Figure 5C,D). In addition several smaller proteins (<25 000) were observed. An analysis of the total proteins synthesized in response to both the 15S and 12S rat casein mRNAs revealed a close correspondence with those proteins specifically immunoprecipitated. The inability to immunoprecipitate selectively more than 60% of the total product specified by the 12S casein mRNA may represent the incomplete recognition of some of the smaller casein fragments since this casein antiserum appears to preferentially recognize the larger molecular weight caseins and these may be more efficiently immunoprecipitated (slots A vs. B and E vs. F of Figure 5). Product analysis was also performed on proteins synthesized in response to the purified 15S mouse casein mRNA. Again several products were observed, one exactly comigrating with the largest of the mouse caseins of 46 000 apparent molecular weight, and a second product migrating at approximately 29 000 to 30 000 coincident with the second mouse casein. In this case, the product was resolved into a doublet on the Laemmli gel system.

In addition to studying the biological purity of the isolated casein mRNAs in the cell-free translation assay, these were further characterized by electrophoresis on 3% agarose-urea gels under conditions which have previously been shown to prevent mRNA aggregation (Figure 6). The 15S rat casein mRNA migrated as a homogeneous RNA doublet with no apparent major contamination with either 18S rRNA or the 12S casein mRNA. The isolated 12S rat casein mRNA also appeared to be free of any major RNA contaminants and migrated as a single, symmetrical RNA peak. The slightly uneven baseline observed in these gel scans was due to differential destaining of the agarose and not discrete RNA species. Similiar profiles were also observed on formamide-containing polyacrylamide gels (data not shown).

An accurate determination of the molecular weights of the purified casein mRNAs was a necessary prerequisite for both the estimation of their analytical complexities by hybridization analysis and for the determination of the length of any nontranslated regions within these mRNAs. Accordingly the molecular weights of the 15S and 12S rat casein mRNAs were determined by electrophoresis on both 3% agarose-urea gels and 4% polyacrylamide gels containing 99% formamide using

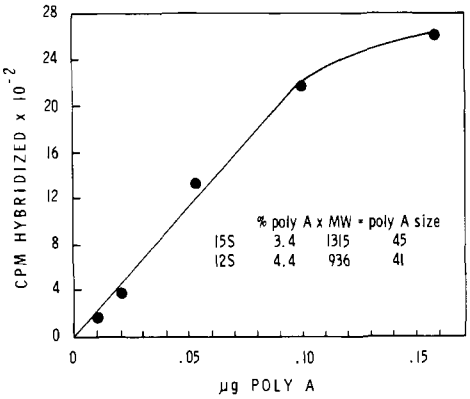

FIGURE 8: Analysis of the poly(A) content in casein mRNA. The percentage of poly(A) present in the purified 15S and 12S casein mRNAs was determined by hybridization with [^{3}H]poly(U) as described under Experimental Procedures and Rosen et al. (1974). Several concentrations of each mRNA were employed and the amount of poly(A) was determined from the standard curve as shown.

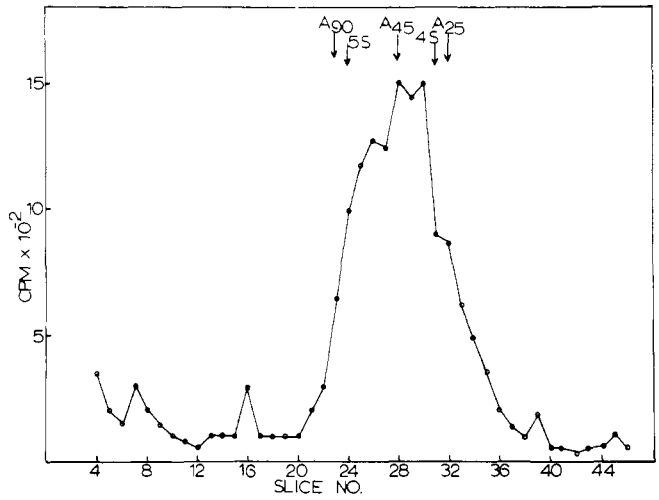

FIGURE 9: Determination of the length of poly(A) in 15S casein mRNA. Purified 15S casein mRNA (4 μg) was subjected to periodate oxidation followed by reduction with [^{3}H]NaBH. Following digestion with a combination of pancreatic and T_1 RNases, the length of the resistant poly(A) fragment was determined on 12.5% polyacrylamide gels in a Tris-citric acid buffer, pH 8.0. Poly(A) standards of known lengths were treated in a similar manner and run on a parallel gel. In addition 5S and 4S rat RNAs were run as internal standards on both gels. The gels were stained with 1% methylene blue as described, cut into 1.5-mm slices, and digested with 0.5 ml of 30% H_2O_2 at 60 °C, and the radioactivity was determined in a Triton–Spectrofluor (1:2) liquid scintillation cocktail. The details of this method are described in Monahan et al. (1976).

several other well-characterized mRNAs as molecular weight standards (Figure 7A,B). A linear relationship was observed when the log of the molecular weights of viral MS-2 RNA (mol wt 1 220 000; Fiers et al., 1976), ovalbumin mRNA (mol wt 650 000; Woo et al., 1975), and hemoglobin mRNA (mol wt 205 000; Spohr et al., 1976) were plotted vs. their respective R_f's. The apparent molecular weight of the 15S doublet under these conditions was 450 000 ± 30 000 or approximately 1315 ± 88 nucleotides, while the 12S casein mRNA was 320 000 ± 25 000 or approximately 936 ± 73 nucleotides. Ribosomal RNAs were not the appropriate standards under these electrophoretic conditions (Figure 7, dashed line) and their use would have resulted in a considerable overestimation of the molecular weights of the casein mRNAs (see Discussion).

Confirmation of these molecular weights for the 15S and 12S casein mRNAs was obtained by determining the percentage of each of the purified mRNAs that was composed of poly(A) by hybridization with [^{3}H]poly(U). A linear poly(A) standard curve was initially established (Figure 8) under conditions of [^{3}H]poly(U) excess which favored triplex formation. Using this specific hybridization assay, 15S casein mRNA was found to contain 3.4% poly(A), while the 12S casein mRNA contained 4.4% poly(A). As shown in Figure 8, the respective lengths of the poly(A) tails for each of these mRNAs would be expected to contain 45 and 41 adenosines using the previously determined molecular weights obtained by gel electrophoresis. The number average size of the poly(A) tail of the 15S casein mRNA was in fact determined to be approximately 42 adenosines (Figure 9). A heterogeneous distribution of poly(A) lengths ranging from 15 to 150 adenosines was obtained in this steady-state population of purified 15S casein mRNA with a number average size of 42 adenosines. Thus, a molecular weight estimation of 1230 nucleotides for the purified 15S casein mRNA would be obtained by poly(A) analysis in reasonable agreement with the value obtained by gel electrophoresis.

Discussion

A 15S rat casein mRNA fraction has been isolated from both rat and mouse lactating mammary tissue. The 178-fold purified mRNA is free of apparent rRNA contamination as judged by agarose–urea gel electrophoresis and contains at least 90% casein mRNA activity when assayed in the wheat-germ, cell-free, translation assay. Two hundred micrograms of the purified rat 15S mRNA fraction was isolated from 850 mg of total lactating RNA. Since 5 mg of total RNA is routinely isolated per g of lactating tissue (Rosen et al., 1975b) and approximately 10 g of tissue is obtained per lactating rat, only 15 to 20 animals were initially required for this isolation. This mRNA fraction was of sufficient purity to generate selective hybridization probes (Rosen and Barker, 1976).

The 15S casein mRNA fraction specified in the wheat-germ, cell-free translation system a product comigrating with the largest authentic rat casein of 42 000 apparent molecular weight and a second product which migrated slightly faster than the 30 000 molecular weight rat casein. Attempts to increase the proportion of the larger rat casein synthesized in vitro by increasing the KCl concentration either prior to or after the initiation of protein synthesis or by translation in a reticulocyte, cell-free, translation assay proved unsuccessful. However, raising the KCl concentration increased the release of immunoprecipitable casein. Similar results have also been reported for several other mRNAs (Schmeckpeper et al., 1974; Vermorken et al., 1975; Benveniste et al., 1976). The limited synthesis of the largest rat casein in vitro may, therefore, reflect either the actual amount of this isolated mRNA present in the 15S mRNA doublet, inefficient chain completion in vitro, or partial proteolysis and processing of the in vitro product. Further experiments will be required to distinguish among these alternatives. The relative migration of these caseins synthesized in vitro and the authentic rat caseins suggests that a significantly larger in vitro product has not been synthesized. The sensitivity of the 25-cm long, 5–20% polyacrylamide gradient slab gels would have permitted the detection of an additional five amino acids or more if they were present in the in vitro product. In fact, the major product specified by the 15S casein mRNA actually migrated slightly faster (apparent mol wt 28 500) than the authentic 29 500 molecular weight casein. A similar observation has also been made for ovalbumin synthesized in vitro (Rosen et al., 1975) and probably reflects the

difference in migration of the native glycoprotein compared with the in vitro product lacking carbohydrate.

The purity of the second casein mRNA species which migrated as a single 12S RNA species during agarose–urea gel electrophoresis was more difficult to ascertain. Although there was a close correspondence of both the total and specifically immunoprecipitated proteins as analyzed by fluorography on slab gels and the total mRNA specific activities were similar for both casein mRNA fractions, only 60% of the radioactive protein synthesized was immunoprecipitable with the total anticasein IgG fraction. This may reflect both the preference of this antibody for the larger rat caseins and its inability to quantitatively precipitate the smaller fragments lacking carbohydrate which are synthesized in vitro. Although the 12S mRNA was only purified 95-fold compared with the initial RNA extract based on the immunoprecipitation data, this fraction represented greater than a 200-fold purification when the kinetics of its cDNA hybridization with the purified mRNA and total RNA extract were compared (Rosen and Barker, 1976). Although the data are suggestive of purity greater than 60%, additional characterization is required to support this conclusion. It should also be noted that the major product specified by the 12S mRNA migrated slightly slower than the smallest rat casein of 25 000 apparent molecular weight. Thus, only in this case is there any presumptive evidence for a precursor that is approximately 1000 daltons larger than the native glycoprotein.

In order to isolate and characterize discrete species of casein mRNA, it was necessary to employ either denaturing or disaggregating conditions during the individual purification procedures. The use of heat denaturation followed by rapid cooling in a low ionic strength buffer prior to dT-cellulose chromatography improved both the recovery and selectivity of this technique for casein mRNA. Similar results have recently been reported for the chromatography of ovalbumin mRNA on dT-cellulose (Rhoads, 1975). Presumably, the increased contamination with 18S rRNA and the decreased recovery of poly(A)-containing mRNA when nondenaturing conditions are employed may reflect hybrid formation between the 5′ end of the mRNA and the 3′ end of the 18S rRNA (Steitz and Jakes, 1975). This type of association would be prevented by heat denaturation and rapid cooling under conditions of low ionic strength and would, therefore, improve the resolution of both the sizing and poly(A)-affinity chromatography techniques.

An estimation of the molecular weights of the purified 12S and 15S rat casein mRNAs of 320 000 ± 25 000 and 450 000 ± 30 000 nucleotides, respectively, was obtained by both a comparison of their migration on agarose–urea gels with several other well-characterized mRNA standards, and by poly(A) analysis. Both mRNA fractions were larger than required to specify their individual protein products even including the addition of poly(A) tails of number average lengths of 42 and 38 adenosines. This additional noncoding information comprises between 15 and 40% of the casein mRNAs and has also been observed in many other purified mRNAs (Williamson et al., 1971; Woo et al., 1975). The exact size and location of these untranslated sequences will require sequence analysis of both the individual rat caseins, as well as their mRNAs.

The determination of RNA molecular weights is now routinely performed by polyacrylamide gel electrophoresis under denaturing conditions (Boedtker, 1971; Pinder et al., 1974; Lizardi et al., 1975; Spohr et al., 1976). However, overestimation of the molecular weights of several mRNAs has been observed, when rRNAs have been used as standards, even when apparent denaturing conditions have been employed (Woo et al., 1975). For a large mRNA, such as ovalbumin mRNA, analysis on either formamide-containing polyacrylamide gels or agarose–urea gels led to a 40% overestimation of the size of this mRNA (900 000 vs. 650 000). This may have resulted from residual secondary structure in the higher GC content rRNAs which is present even in 6 M urea or 90% formamide. Such GC-rich structures in 28S rRNA have been observed in the electron microscope even in the presence of 70% formamide and 4 M urea (Wellauer et al., 1974).

Recent studies by Spohr et al. (1976) have suggested that full denaturation of double-stranded RNA is accomplished during electrophoresis on formamide-containing polyacrylamide gels only when the electrophoresis is performed at 55 °C. Heat denaturation of mRNA in formamide prior to electrophoresis and formaldehyde treatment have also been utilized to fully denature RNA (Boedtker, 1971; Lizardi et al., 1975). In order to circumvent this problem of secondary structure in rRNA standards, we have used several well-characterized mRNA standards of comparable physical-chemical properties (Van et al., 1976) to determine the molecular weights of the individual casein mRNA fractions. The validity of this approach was confirmed by an independent method which relied on the determination of both the length of poly(A) and the percentage of casein mRNA containing poly(A) (Woo et al., 1975).

The length of the poly(A) sequences in the purified 12S and 15S casein mRNA fractions is consistent with the steady-state length of poly(A) found in ovalbumin mRNA (Woo et al., 1975), hemoglobin mRNA (Morrison et al., 1973), and HeLa mRNA (Sheiness et al., 1975). Moreover, the heterogeneous distribution in poly(A) size observed for casein mRNA supports the hypothesis that a random endonucleolytic attack is involved in the poly(A) shortening observed during the aging of mRNA (Sheiness et al., 1975). The presence of extremely short poly(A) tails (<20 adenosine's) presumably accounts for the lack of binding of a significant amount (20 to 50%) of the casein mRNA activity in the lactating RNA extract during the initial dT-cellulose chromatography procedure (Rosen et al., 1975b).

The isolation and preliminary characterization of two rat casein mRNA fractions have been reported. Both the physical and biological purity of these isolated mRNAs appears to be sufficient to permit their further chemical characterization and to allow the generation of selective complementary DNA hybridization probes (Rosen and Barker, 1976).

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References

Aviv, H., and Leder, P. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1408.

Benveniste, K., Wilczek, J., Ruggier, A., and Stern, R. (1976), *Biochemistry 15,* 830.

Boedtker, H. (1971), *Biochim. Biophys. Acta 240,* 448.

Boedtker, H., Crkvenjakov, R. B., Dewey, K. F., and Lanks, K. (1973), *Biochemistry 12,* 4356.

Bonner, W. M., and Laskey, R. A. (1974), *Eur. J. Biochem. 46,* 83.

Both, B. W., Furuichi, Y., Muthukrishnan, S., and Shatkin,

A. J. (1975), *Cell 6,* 185.
Burr, H., and Lingrel, J. (1971), *Nature (London), New Biol. 233,* 41.
Favre, A., Bertazzoni, U., Berns, A. J., and Bloemendal, H. (1974), *Biochem. Biophys. Res. Commun. 56,* 273.
Fiers, W., Contreras, R., Duerinck, F., Haegeman, G., Iserentant, D., Merregaert, J., Min Jou, W., Molemans, F., Raeymaekers, A., Van den Berghe, A., Volckaert, G., and Ysebaert, M. (1976), *Nature (London) 260,* 500.
Gaye, P., and Houdebine, L. M. (1975), *Nucleic Acid Res. 2,* 707.
Gielen, J., Aviv, H., and Leder, P. (1974), *Arch. Biochem. Biophys. 163,* 146.
Haines, M. E., Carey, N. H., and Palmiter, R. D. (1974), *Eur. J. Biochem. 43,* 146.
Houdebine, L. M., and Gaye, P. (1976), *Eur. J. Biochem. 63,* 9.
Laemmli, U. (1970), *Nature (London) 227,* 280.
Lizardi, P. M., Williamson, R., and Brown, D. D. (1975), *Cell 4,* 199.
Mandal, H., Sutton, A., Chen, V.-J., and Sarker, S. (1974), *Biochem. Biophys. Res. Commun. 56,* 988.
Monahan, J. J., Harris, S. E., and O'Malley, B. W. (1976), *J. Biol. Chem. 251,* 3738.
Morrison, M. R., Brinkley, S. A., Gorski, J., and Lingrel, J. B. (1974), *J. Biol. Chem. 249,* 5290.
Morrison, M., Merkel, C. G., and Lingrel, J. B. (1973), *Mol. Biol. Rep. 1,* 55.
Pinder, J. C., Staynov, D. Z., and Gratzer, W. B. (1974), *Biochemistry 13,* 5373.
Rhoads, R. (1975), *J. Biol. Chem. 250,* 8088.
Roberts, B. E., Gorecki, M., Mulligan, R. C., Danna, K. J., Rozenblatt, S., and Rich, A. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1922.
Rosen, J. M., and Barker, S. W. (1976), *Biochemistry 15* (following paper in this issue).
Rosen, J. M., Harris, S. E., Rosenfeld, G. C., Liarakos, C. D., and O'Malley, B. W. (1974), *Cell Differ. 3,* 103.
Rosen, J. M., Woo, S. L. C., and Comstock, J. P. (1975b), *Biochemistry 14,* 2895.
Rosen, J. M., Woo, S. L. C., Holder, J. W., Means, A. R., and O'Malley, B. W. (1975a), *Biochemistry 14,* 69.
Rosen, J. M., Woo, S. L. C., Means, A. R., and O'Malley, B. W. (1976), *Methods Mol. Biol. 8,* 369.
Schmeckpeper, B. J., Cory, S., and Adams, J. M. (1974), *Mol. Biol. Rep. 1,* 355.
Sheiness, D., Puckett, L., and Darnell, J. E. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1077.
Spohr, G., Mirault, M.-E., Imaizumi, T., and Scherrer, K. (1976), *Eur. J. Biochem. 62,* 313.
Stavnezer, J., Huang, R. C. C., Stavnezer, E., and Bishop, J. M. (1974), *J. Mol. Biol. 88,* 43.
Steitz, J. A., and Jakes, K. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4734.
Suzuki, Y., and Brown, D. D. (1972), *J. Mol. Biol. 63,* 409.
Taborsky, G. (1974), *Adv. Protein Chem. 28,* 91.
Van, N. T., Holder, J. W., Woo, S. L. C., Means, A. R., and O'Malley, B. W. (1976), *Biochemistry 15,* 2054.
Vermorken, A. J. M., Kilderink, J. M. H. C., Van De Ven, W. J. M., and Bloemendal, H. (1975), *Biochim. Biophys. Acta 414,* 167.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Wellauer, P. K., Dawid, I. B., Kelley, D. E., and Perry, R. P. (1974), *J. Mol. Biol. 89,* 397.
Williamson, R., Morrison, M., Lanyon, G., Eason, R., and Paul, J. (1971), *Biochemistry 10,* 3014.
Woo, S. L. C., Rosen, J. M., Liarakos, C. D., Choi, Y. C., Busch, H., Means, A. R., and O'Malley, B. W. (1975), *J. Biol. Chem. 250,* 7027.