# Regulation of Casein Messenger RNA during the Development of the Rat Mammary Gland†

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ABSTRACT: Casein mRNA was isolated and partially purified from RNA extracts of rat lactating mammary glands and translated in a heterologous cell-free protein synthesizing system derived from wheat germ. Casein mRNA activity was assayed by immunoprecipitation using a specific antiserum prepared against a mixture of the purified rat caseins. Properties of rat casein mRNA were examined using a variety of sizing techniques, including chromatography on Sepharose 4B, sedimentation on sucrose gradients after heat denaturation, and electrophoresis on 2.5% agarose gels in 6 *M* urea. Casein mRNA activity was found in an 8–16S region after gradient centrifugation with the peak occurring at 10.5 S. In addition, the binding of rat casein mRNA to dT-cellulose was examined. Only 40% of the total casein mRNA activity was selectively retained. A partial purification of casein mRNA was accomplished by a combination of these sizing and affinity chromatography techniques. In the purified preparations casein mRNA activity comprises approximately 90% of the total mRNA activity. Characterization of this material by agarose gel electrophoresis revealed two main bands of RNA at approximately 12 and 16 S, both containing casein mRNA activity. These mRNAs were of the correct size to code for two of the principal rat caseins of approximately 25,000 and 42,000 molecular weights. Casein mRNA and total mRNA activities were then compared in total RNA extracts at various stages of normal mammary gland development in the rat, i.e. during pregnancy, lactation, and involution following weaning. A selective induction of casein mRNA activity compared to total mRNA activity was found to occur during pregnancy and lactation. Moreover, a selective loss of activity was also observed during mammary gland involution. A surprisingly high level of casein mRNA activity was found in RNA extracts from early and midpregnant mammary glands.

The growth and differentiation of the mammary gland are controlled by the multiple interactions of several peptide and steroid hormones (Turkington, 1971). Furthermore, the structure and function of both normal and malignant mammary epithelial tissues are under hormonal regulation (Hilf et al., 1967). Because of the complexity of eukaryotic DNA it is necessary to have specific biochemical markers in order to study the regulation of differentiated function at the molecular level. In the mammary gland, casein synthesis has been widely used as such a specific indicator of differentiated function (Juergens et al., 1965). Casein is actually a group of proline-rich, acidic phosphoproteins which comprise between 70 and 80% of rat milk protein (Jenness, 1974). As with most secretory proteins, casein is synthesized on bound polyribosomes (Gaye et al., 1973b). Although casein synthesis has been monitored under a variety of physiological and experimental conditions both in vivo and in vitro (Lockwood et al., 1966) relatively little is known about the factors regulating casein mRNA transcription and translation. Some progress has recently been made in this area. In a preliminary report (Gaye et al., 1973b) $\alpha_s$-casein mRNA was isolated from the bound polyribosomes of the ewe mammary gland. More recently a total mRNA fraction has been isolated from the ewe mammary gland that contains $\alpha_s$-, $\beta$-, and $\kappa$-casein mRNA activity (Gaye et al., 1973a).

Because of our interest in studying the hormonal regulation of both normal and malignant mammary tissue, we have extended these studies to the rat and have isolated and partially characterized rat casein mRNA. In order to better understand the regulation of differentiated function in the mammary gland we have used the wheat germ translation system to compare casein mRNA activity and total tissue mRNA activity during various stages of mammary gland development. The partial purification of rat casein mRNAs was accomplished by a combination of sizing techniques and affinity chromatography using dT-cellulose. The techniques described should permit the isolation of adequate quantities of purified casein mRNA to generate specific hybridization probes and to allow further characterization of each of the rat casein mRNAs.

## Experimental Procedures

*Animals.* The entire thoracic and abdominal mammary glands were surgically removed under ether anesthesia from 8-week-old (approximately 200 g) female Charles River or Texas Inbred rats. The lymph nodes were then removed and the glands rinsed in cold 0.9% NaCl and quickly frozen at −80°. All tissue was stored in a liquid nitrogen freezer at −196° until the extraction of RNA was performed. For developmental studies, day zero of pregnancy is defined as the day on which copulation plugs were found and the day of birth is designated as the first day of lactation.

*Casein Isolation and Characterization.* Milk was collected from female Charles River rats, 5 to 7 days post-partum, by the atraumatic vacuum technique of Young and Nelstrop (1970). Whole casein was obtained by isoelectric precipitation of skim milk at pH 4.6 and 20° by the procedure of McMeekin et al. (1959). Following reprecipitation, and several washings with water, the casein was dried by successive extraction with ethanol, acetone, and ether. The individual rat caseins were isolated by chromatography on DEAE-52-cellulose in the presence of 3.3 *M* urea–0.01 *M*

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Table I: Specificity of Casein Immunoprecipitation.

| Sample | Total mRNA Act.[a] (cpm − BG) | Casein mRNA Act.[b] (cpm − BG) | Casein mRNA/ Total mRNA (%) |
|---|---|---|---|
| 8-Day lactating mRNA | | | |
| + casein Ab, + casein | 286,000 | 111,000 | 38.7 |
| + casein Ab, − casein | 286,800 | 1,400 | 0.5 |
| + ovalbumin Ab, + ovalbumin | 286,800 | 2,600 | 0.9 |
| Ovalbumin mRNA | | | |
| + casein Ab, + casein | 276,900 | 9,500 | 3.4 |

[a] Background (BG) subtracted when no mRNA added = 43,800 cpm/100 μl assay. [b] Background subtracted when no mRNA added = 1,300 cpm/100 μl assay.

imidazole-HCl buffer at pH 7.0, containing 1 ml of 2-mercaptoethanol per 100 ml and 0.001 *M* $Na_2EDTA$ to prevent casein aggregation and micelle formation (Thompson, 1966). Rechromatography of each major peak was performed on DEAE-cellulose in the same buffer. Protein content in the peak fractions was then analyzed by electrophoresis on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate and 0.001 *M* dithiothreitol using the procedure of Weber and Osborn (1969). Peak fractions containing a single molecular weight species were pooled, dialyzed against 0.01 *M* $NH_4HCO_3$, and lyophilized. A preliminary amino acid analysis of each of the pooled fractions was performed on a Beckman automatic amino acid analyzer Model 121 after hydrolysis for 24 hr at 110° in 6 *N* HCl containing 1% phenol.

A specific rabbit antiserum was prepared against an equal mixture (by weight) of each of the rat caseins bands 1, 2, and 3 (see Figure 2). Immunoprecipitation of rat whey proteins or bovine α-casein was not detected with this antiserum.

*RNA Isolation.* RNA was extracted from whole tissue by direct homogenization of the pulverized frozen mammary glands in a phenol–sodium dodecyl sulfate buffer at pH 8.0 essentially as previously described (Rosen et al., 1975). Because of the large amounts of lipid in the mammary gland a second extraction of the aqueous phase and interphase was performed using a 1:1 mixture of buffer-saturated phenol and chloroform (v/v) instead of phenol alone. The resulting emulsion was then homogenized in a Dounce homogenizer prior to centrifugation to allow total phase separation. The final RNA extracts were reprecipitated at least three times, dissolved in water, and centrifuged at 20,000*g* at 4° to remove any insoluble material. Each RNA preparation had an $A_{260}/A_{280}$ of 2.0 or greater.

*Wheat Germ Cell-Free Translation Assay.* The preparation of the wheat germ S-30 fraction and the components of the assay have previously been described in detail (Rosen et al., 1975). One-hundred microliter assays were routinely employed with a 2-hr incubation at 25°. Four microliters of L-[5-$^3$H]proline (Schwarz/Mann, either 17 or 32.3 Ci/mmol) was added as the radioactive amino acid in the translation assay, since casein is unusually rich in proline (see Results). Following the 2-hr incubation 20 μl of the incubation mixture was removed to determine total mRNA activity as described (Rosen et al., 1975), while the remaining 80 μl was used for specific immunoprecipitation of casein as follows: 20 μl of a solution containing 10% Triton X-100, 0.05 *M* proline, 0.05 *M* sodium phosphate, and 0.7 *M* NaCl, pH 7.5, was added, followed by 0.4 μg of a mixture of purified rat casein bands 1, 2, and 3 and 20 μl of casein antiserum. Following incubation at 25° for 30 min the samples were stored at 4° for at least 96 hr. The mixtures were then layered over a 1-ml cushion of 1 *M* sucrose containing wash buffer (2% Triton X-100–0.01 *M* proline–0.01 *M* sodium phosphate–0.14 *M* NaCl (pH 7.5)) in 10 × 75 mm ignition tubes at 4°. The tubes were centrifuged at 2000*g* for 10 min at 4°, and the solution on top of the sucrose layer was removed. The surface of the sucrose layer was then rinsed twice with the cold wash buffer and the sucrose layer carefully removed. The immunoprecipitate was rinsed an additional two times with cold wash buffer, dissolved in 0.1 ml of NCS solubilizer (Amersham–Searle), and counted in 5.0 ml of a toluene–Spectrofluor scintillation cocktail. This extensive washing procedure was utilized to minimize nonspecific trapping during immunoprecipitation (see Table I). Product analysis was performed by sodium dodecyl sulfate gel electrophoresis as previously described (Rosen et al., 1975).

*Casein mRNA Isolation and Characterization.* The procedures used for the purification of casein mRNA involved a combination of sizing techniques and selective adsorption of poly(A) containing mRNA to either nitrocellulose filters or dT-cellulose. These techniques have been successfully employed to purify ovalbumin mRNA (Rosen et al., 1975) and were used to purify and characterize casein mRNA with the following modifications. Casein mRNA was characterized by electrophoresis on 2.5% agarose gels (Bio-Rad, electrophoresis grade) containing 6 *M* urea. Extraction of mRNA activity from the gels was performed by homogenization in cold 0.1 *M* $NaC_2H_3O_2$ buffer (pH 5.0). Prior to RNA precipitation 1 vol of 100% ethanol was added to the combined aqueous layers and the solution stored at −20° for 1 hr. Following centrifugation at 20,000*g* for 15 min, the aqueous upper layer was removed from any pelleted agarose and brought to 0.5 *M* $Na^+$. An additional volume of 100% ethanol was added and the RNA precipitated by storage at −20° overnight. The RNA was pelleted by centrifugation at 20,000*g* for 30 min and dissolved in 20 μl of $H_2O$.

Analysis of RNA by sucrose gradient centrifugation was performed under partially denaturing conditions previously shown to prevent mRNA aggregation (Rosen et al., 1975; Haines et al., 1974). RNA was dissolved in 0.001 *M* $Na_2EDTA$ (pH 5.0) and denatured for 30 sec at 70° followed by quick cooling to 4°. Samples were then layered on 0.3–1.0 *M* sucrose gradients (Rosen et al., 1974). Following centrifugation, 10-drop fractions were collected, brought to 0.25 *M* NaCl, and precipitated with 2 vol of ethanol. The pelleted RNA fractions were each dissolved in 50 μl of water and 10 μl of each fraction was assayed in the wheat germ translation system.

## Results

While bovine casein has been extensively studied and its individual components purified, little has been done to analyze rat casein other than starch gel electrophoresis after $Ca^{2+}$ and rennin precipitation (Juergens et al., 1965). The purification and limited characterization of rat casein were necessary prerequisites for further studies on casein mRNA.

The separation of the components of rat casein was accomplished by chromatography of total acid-precipitated casein on DEAE-cellulose in the presence of urea and mer-

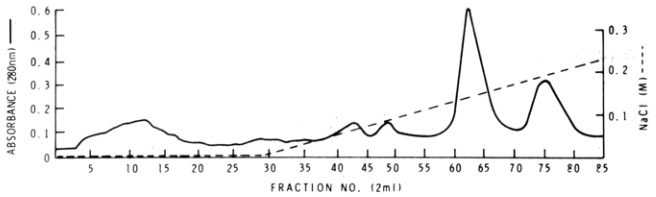


FIGURE 1: DEAE chromatography of acid-precipitated rat casein. Lyophilized rat casein (17 mg) was dissolved in 20 ml of 3.3 *M* urea–0.01 *M* $Na_2EDTA$–0.01 *M* imidazole buffer (pH 7.0) containing 0.2 ml of 2-mercaptoethanol at room temperature, passed through a Millipore 0.45-$\mu$ filter, and applied to a 9 mm × 15 cm DEAE-52 column equilibrated in the same buffer. After the absorbancy had returned to the base line, the rat casein bands were eluted with a 0 to 0.35 *M* linear NaCl gradient in the urea buffer (60 ml in each reservoir).

captoethanol to minimize casein aggregation (Figure 1). Contaminating whey proteins in the pH 4.6 fraction and a small amount of band 2 casein (see Figure 2) did not bind to the DEAE-cellulose. Four major protein peaks were then eluted by a linear NaCl gradient. The doublet eluting at approximately 0.05 and 0.08 *M* NaCl was found to be composed of a single molecular weight species as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (data not shown). The two species probably differ only in their extent of phosphorylation and/or in their carbohydrate content and were, therefore, pooled for future studies. Each of the peaks was rechromatographed on DEAE-cellulose and the peak fractions were then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 2). The gels were overloaded in order to detect the presence of minor contaminants. Each of the pooled fractions was composed of an essentially homogeneous molecular weight protein species of an estimated 95% purity or greater.

The three casein species were designated bands 1, 2, and 3 based on the determination of their apparent molecular weights by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Band 1 had an apparent molecular weight of 42,000 and represented the major peak on the DEAE chromatogram, eluting at 0.15 *M* NaCl. Band 2 had an apparent molecular weight of 30,000 and chromatographed as the initial doublet eluting at 0.05 and 0.08 *M* NaCl on DEAE-cellulose. Finally, band 3 had an apparent molecular weight of 25,000 and eluted at approximately 0.2 *M* NaCl during DEAE chromatography. A preliminary amino acid analysis was performed on each of the rat casein fractions (data not shown). All three were highly acidic proteins, rich in glutamic acid, and contained characteristically high amounts of proline and serine and a low content of cystine found in bovine and sheep casein. However, differences in the phenylalanine to tyrosine ratio and in the amounts of alanine and isoleucine suggested that they were distinct protein species. Since our predominant interest was in studying the regulation of casein mRNA our attention was now directed toward the isolation and translation of casein mRNA. The individual rat caseins were pooled and used to generate a specific antiserum in rabbits. In addition they were used separately as markers for product analysis of the immunoprecipitated protein in the wheat germ assay.

In initial experiments casein mRNA activity was determined in RNA preparations isolated from 8-day lactating rat mammary glands, which has been enriched for poly(A)-containing mRNA by adsorption to nitrocellulose filters. When this RNA was added to the wheat germ cell-free translation assay it was found that approximately 39% of the total mRNA activity could be accounted for by casein

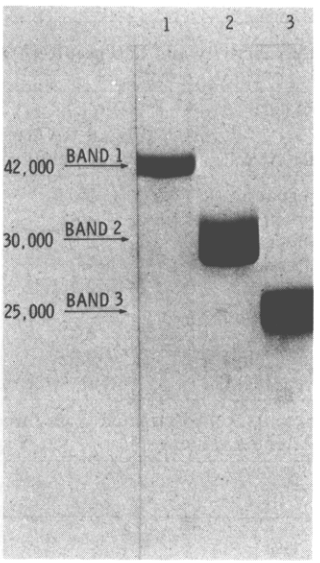


FIGURE 2: Gel electrophoresis of DEAE rat casein peaks. Sodium dodecyl sulfate gel electrophoresis was performed according to Weber and Osborn (1969) on 10% polyacrylamide gels. From left to right: gel 1, DEAE fraction 63; gel 2, DEAE fractions 42 and 48; gel 3, DEAE fraction 75. The molecular weights of each of the caseins were determined relative to other known proteins and are shown by the arrows.

mRNA activity (Table I). The specificity of the immunoprecipitation reaction was determined by several control experiments. In the first experiment an ovalbumin antiserum and purified ovalbumin were added to the assay and only 0.9% of the total radioactivity was trapped in this antibody–antigen complex. In the second control casein carrier was omitted from the immunoprecipitation reaction and only 0.5% of the radioactivity was pelleted through the sucrose cushion. Finally, a partially purified preparation of ovalbumin mRNA was added to the assay and a comparable amount of radioactive protein synthesized. Only 3.4% of this radioactivity was found trapped in the carrier casein antigen–antibody complex. Accordingly, this small extent of trapping was subtracted from all future results. The extent of trapping during immunoprecipitation could be further reduced to less than 1% of the total radioactive protein synthesized if the ribosomes were removed by centrifugation and the immunoprecipitation reaction performed only on released protein in the postribosomal supernatant (data not shown). Although this step may be essential when the specific mRNAs to be assayed represent a small fraction of the total mRNA, it was unnecessary, however, for the accurate determination of casein mRNA activity.

As additional evidence that our immunoprecipitated protein was in fact casein, the casein antigen–antibody complex was solubilized and the radioactive peptides characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 3D). One major peak of radioactivity was found migrating between the rat casein band 2 and band 3 markers of mol wt 25,000 and 30,000 and a minor band was found comigrating exactly with the rat casein band 1 marker of mol wt 42,000. The specificity of the assay was also tested by analyzing the nonspecifically trapped radioactivity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figures 3A and B). The dashed line in Figure 3A represents a uniform background of less than 100 cpm per slice when no mRNA was added to the wheat germ assay indicating the low level of endogenous protein synthesis present in the assay. Moreover, when the ovalbumin

Table II: Total mRNA Activity and Casein mRNA Activity during Mammary Gland Development.

| Total mRNA Act. | Yield (mg of RNA/g of Tissue) | Total mRNA[a] Act. (cpm/μg) | Casein mRNA Act. (cpm/μg) | Casein mRNA/ Total mRNA (%)[b] | Total Casein mRNA Act. (cpm/g of Tissue) |
|---|---|---|---|---|---|
| 5-Day pregnant | 1.6 | 22,000 | 1,100 | 5.0 | 1,700 |
| 10-Day pregnant | 2.4 | 34,200 | 3,900 | 11.4 | 9,400 |
| 12-Day pregnant | 2.0 | 28,300 | 5,500 | 19.4 | 11,000 |
| 13-Day pregnant | 3.7 | 26,200 | 4,100 | 15.6 | 15,000 |
| 20-Day pregnant | 2.7 | 45,500 | 11,500 | 25.3 | 31,000 |
| 2-Day lactating | 5.2 | 53,300 | 21,500 | 40.3 | 112,000 |
| 8-Day lactating | 4.4 | 51,000 | 22,500 | 44.1 | 99,000 |
| Regressed (7 days after weaning) | 0.95 | 30,000 | 500 | 1.7 | 500 |

[a] Each value represents RNA extracted from three to five animals. Specific activities were determined from the linear portions of the translation assay. [b] Uncorrected values.

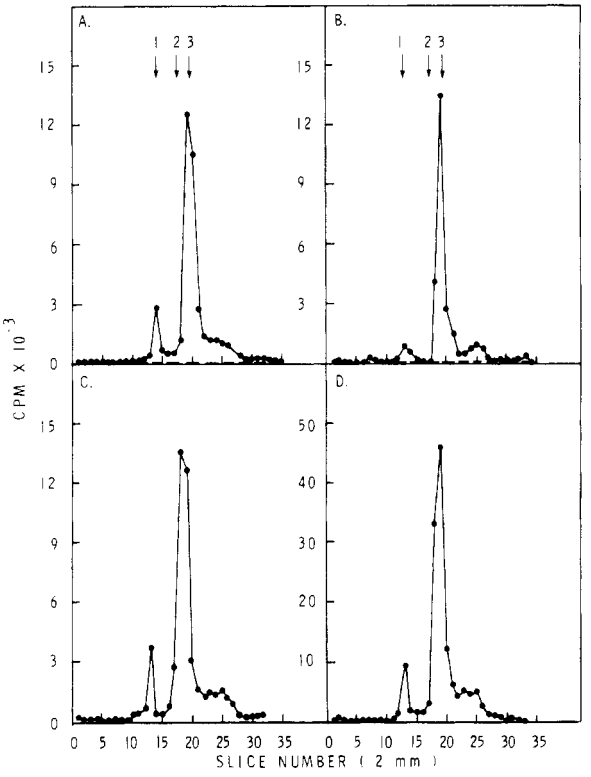


FIGURE 3: Product analysis of immunoprecipitable casein synthesized in the wheat germ assay. Conditions of the assay and immunoprecipitation were as described in the Experimental Procedures section and by Rosen et al. (1975): (A) 10-day pregnant nitrocellulose-adsorbed RNA (●, 3.3 μg); no mRNA added, control (---); (B) 13-day pregnant nitrocellulose-adsorbed RNA (●, 4.2 μg); ovalbumin mRNA trapping control (---, 3 μg); (C) 20-day pregnant nitrocellulose-absorbed RNA (3.1 μg); (D) 8-day lactating nitrocellulose-adsorbed RNA (3.1 μg). The arrows represent the positions of the rat casein markers run as internal standards.

mRNA trapping control was performed as previously described, a gel background of less than 200 cpm per slice was routinely observed. This represented less than 2% of the peak radioactivity in all cases.

Having established the specificity of the assay we analyzed nitrocellulose-bound RNA preparations isolated from the rat mammary gland at different stages of development. Poly(A) containing RNA isolated from 10-, 13-, and 20-day pregnant rat mammary glands was assayed for casein mRNA activity. Somewhat surprisingly it was found that between 15 and 25% of the total mRNA activity in these preparations was composed of casein mRNA activity (data not shown). The immunoprecipitated proteins synthesized in response to these exogenous mRNAs were also characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Figures 3A–C). The pattern of radioactive proteins found after gel electrophoresis was identical with that obtained using filter-bound 8-day lactating mRNA. This indicated that considerable amounts of biologically active casein mRNA existed in the rat mammary gland prior to parturition.

In order to obtain a more quantitative estimate of the variations in both casein mRNA activity and total mRNA activity during mammary gland differentiation total RNA preparations obtained from pregnant, lactating, and regressed mammary glands were assayed in the wheat germ translation system (Table II). Glands were pooled from three to five animals in each case. Several concentrations of each total RNA preparation were assayed and the specific activities determined from the initial linear portions of the assay. Total RNA preparations were utilized rather than nitrocellulose-bound or dT-cellulose bound RNA in order to avoid any preferential losses of either total mRNA or casein mRNA during partial purification. However, a qualitatively similar pattern was observed for both casein mRNA and total mRNA activities when partially purified RNA preparations were assayed (data not shown). Thus, these results suggest that the twofold increase in total mRNA specific activity and the 20-fold increase in casein mRNA specific activity that are observed between 5 days of pregnancy and 2 days of lactation (Table II) represent an actual increase in the mRNA concentration. Casein mRNA increases from approximately 5 to 45% of the total mRNA activity during mammary gland differentiation. After mammary gland regression following weaning there is a rapid loss in casein mRNA activity to essentially zero, while total mRNA activity is reduced to a value comparable to the 5-day pregnant mammary RNA extract. Furthermore, in a preliminary experiment no casein mRNA activity was detected in the RNA extracts of two hormone-dependent DMBA-induced rat mammary tumors (data not shown). When the selective increase in casein mRNA activity is corrected for a two- to threefold increase in RNA recovery during mammary gland development it is apparent that there is approximately a 60-fold increase in total casein mRNA activity that occurs during pregnancy and lactation. However, during mammary gland development the percentage of fat cells is markedly reduced and the glandular tissue may represent as much as 75% of the total gland during lactation (Munford, 1963). Since histological examination of these tissue

Table III: Partial Purification of Casein mRNA.

| | RNA (mg) | Total mRNA Act. (cpm/μg) | Casein mRNA Act. (cpm/μg) | Casein mRNA/ Total mRNA (%)[a] | Fold |
|---|---|---|---|---|---|
| Total nucleic acid extract | 159 | 37,500 | 12,700 | 53.8 | |
| Sepharose peak no. 1 | 9 | 70,600 | 31,100 | 64.0 | 2.5 × |
| dT-Cellulose | 0.58 | 347,900 | 197,100 | 76.6 | 16.0 × |
| Sucrose gradient peak | 0.058 | 657,800 | 453,900 | 89.0 | 36.0 × |

[a] These values have been corrected for a 20% loss during the immunoprecipitation procedure assayed using an $^{125}$I-labeled casein internal standard.

preparations was not performed these results cannot be accurately quantitated as the amount of casein mRNA activity per epithelial cell. However, the following general correlations can be made based on previous reports of mammary gland development (Munford, 1963). Thus, when the casein mRNA activity in Table II is expressed relative to the percentage of glandular tissue the increase in total casein mRNA is only 8- to 11-fold from day 5 of pregnancy until lactation. This change in casein mRNA activity is even less dramatic if the lactating RNA preparations are compared to midpregnant (10 to 13 day) RNA extracts, i.e. only a three- to fourfold increase. Therefore, the presence of appreciable amounts of biologically active casein mRNA early in pregnancy and prior to lactation suggests that the regulation of casein synthesis and/or secretion may be influenced by factors other than the tissue levels of casein mRNA (see Discussion).

Since casein mRNA activity comprised a large percentage of the total mRNA activity in the lactating rat mammary gland it should be possible to purify the individual casein mRNAs by a combination of sizing and affinity chromatographic procedures. Such procedures have previously been employed in our laboratory to purify ovalbumin mRNA which also comprises between 30 and 45% of the total mRNA activity in a whole tissue RNA extract (Rosen et al., 1975). Chromatography of a total lactating gland RNA extract on Sepharose 4B results in the removal of any contaminating DNA, 28S rRNA, and 5S and 4S RNA from the casein mRNAs (Figure 4). Two peaks of total mRNA activity are seen when the Sepharose 4B fractions are assayed in the wheat germ translation system. The majority of the casein mRNA activity coincides with the peak of total mRNA activity preceding the 18S rRNA. However, a small amount of casein mRNA activity also elutes with the second mRNA activity peak. When the proteins synthesized in response to mRNA from the second peak are analyzed by sodium dodecyl sulfate gel electrophoresis they consist of proteins in the 15,000 to 23,000 mol wt range (data not shown). Furthermore, radioactive valine is incorporated into these proteins in the cell-free system to a greater extent than proline. These results are compatible with the hypothesis that the second peak of mRNA activity contains the mRNA for α-lactalbumin. However, this observation should be confirmed by a specific immunoprecipitation assay and further product analysis. Thus, chromatography of the total mammary gland RNA extract on Sepharose 4B allows a partial purification of the casein mRNAs from other mammary gland mRNAs, as well as from DNA, 28S rRNA, and 4S RNA. The specific activity of casein mRNA is enriched approximately 2.5 times and the percentage of the total mRNA activity increases 10% (Table III).

Further purification of poly(A) containing RNA is then accomplished by chromatography of the Sepharose 4B peak

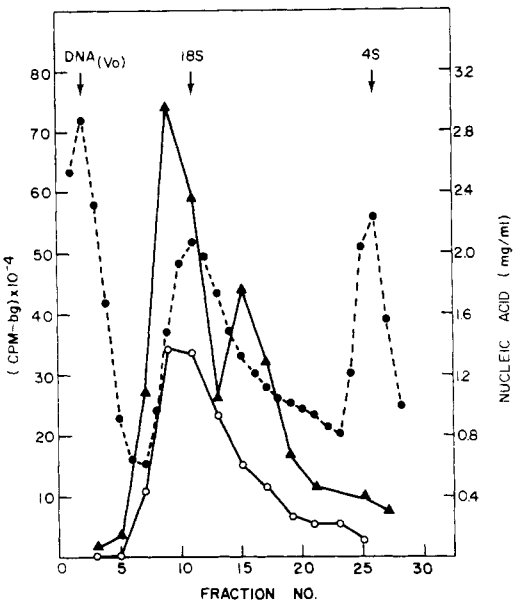


FIGURE 4: Sepharose 4B chromatography of total lactating nucleic acid. Seventy milligrams of a pH 8.0 phenol-sodium dodecyl sulfate total nucleic acid extract of pooled 8–10-day lactating mammary glands was applied to a 5 cm × 100 cm Sepharose 4B column run in 0.1 *M* NaOAc-0.001 *M* $Na_2EDTA$ (pH 5.0) buffer. Forty-milliliter fractions were collected and precipitated by the addition of NaCl to 0.4 *M* and 2 vol of ethanol. The RNA was collected by centrifugation and dissolved in 1.0 ml of $H_2O$ and the $A_{260}$ of each fraction was determined. Ten microliters of each fraction was assayed in the wheat germ system exactly as described under Experimental Procedures: total mRNA activity (▲–▲); casein mRNA activity (○–○); nucleic acid concentration (●---●).

of casein mRNA activity on dT-cellulose. This results in an additional sevenfold increase in casein mRNA activity, which now represents approximately 57% of the total mRNA activity. Thus, a partial purification of casein mRNA activity occurs during dT-cellulose chromatography. During this procedure it is routinely observed that only 40% of the casein mRNA activity and 30% of the total mRNA activity can be bound to the dT-cellulose. A similar observation is made when the total RNA extract is directly chromatographed on dT-cellulose without prior fractionation on Sepharose 4B. As expected, 6.2% of the partially purified Sepharose peak RNA fraction selectivity binds to dT-cellulose (Table III) while on the average only 2% of the total RNA extract will bind (data not shown). This failure of 60% of the casein mRNA activity and 70% of the total mRNA activity to bind to dT-cellulose is not the result of column overloading, since little or no binding of the flow-through RNA is observed on reapplication after thoroughly equilibrating the column. The Sepharose-dT purified RNA is analyzed by centrifugation on 0.3–1.0 *M* sucrose gradients after heat denaturation to prevent mRNA aggregation

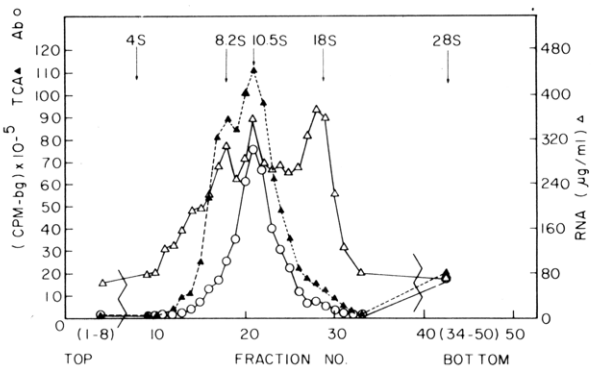


FIGURE 5: Sucrose gradient analysis of Sepharose 4B- and dT-cellulose purified casein mRNA. Approximately 275 $\mu$g of RNA was centrifuged after heating on 0.3–1.0 *M* sucrose gradients in two buckets of a Beckman SW40 rotor at 35,000 rpm for 16 hr as described under Experimental Procedures: RNA concentration (Δ–Δ); total mRNA activity (▲---▲); casein mRNA activity (O–O). The arrows at 4, 18, and 28 S represent the positions of marker RNAs run on a parallel gradient.

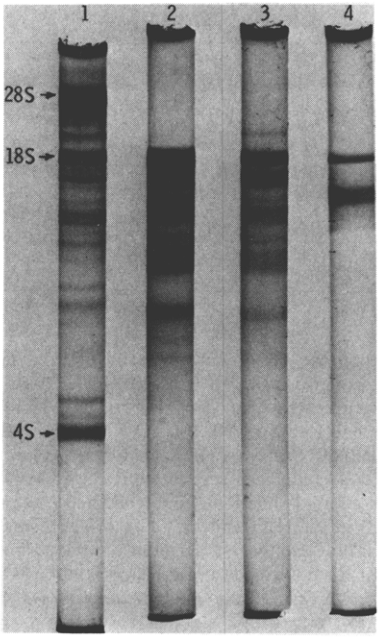


FIGURE 6: Agarose gel electrophoresis of mammary gland RNA. The fractions described in Table III were analyzed by electrophoresis on 2.5% agarose gels. Following electrophoresis gels were stained with a 1% Methylene Blue–15% acetic acid solution as described (Rosen et al., 1974). From left to right: (1) total 8-day lactating mammary gland RNA extract (30 $\mu$g); (2) Sepharose 4B mRNA peak 1 containing the bulk of casein mRNA activity (20 $\mu$g); (3) Sepharose-dT bound RNA (9 $\mu$g); (4) sucrose gradient, peak fraction of casein mRNA activity (4 $\mu$g).

(Figure 5). A broad peak containing both casein mRNA activity and total mRNA activity is detected sedimenting from 8 to 16 S with the maximum casein mRNA activity occurring at 10.5 S. These activities correspond to a small peak of absorbance at 260 nm. An additional shoulder of both total mRNA activity and absorbance is observed at 8.2 S. Approximately one-third of the RNA absorbance is composed of 18S rRNA. The gradient peak activity fraction represents a 36-fold increase in casein mRNA activity over the starting total RNA extract and casein mRNA activity now comprises almost 70% of the total mRNA activity (Table III). This purification scheme is also illustrated by the analysis of each RNA fraction by electrophoresis on

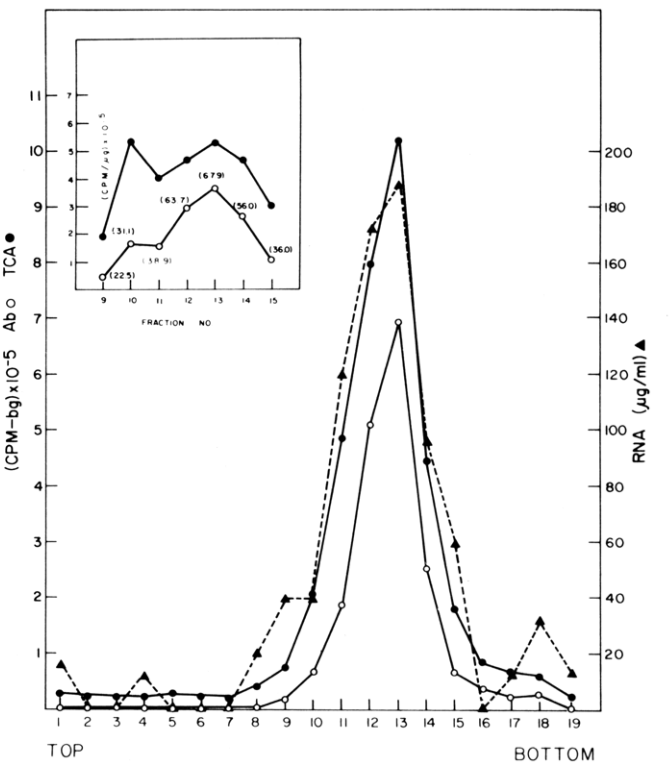


FIGURE 7: Resedimentation of partially purified casein mRNA. Fractions 18–22 of the gradient depicted in Figure 5 were centrifuged again after heat denaturation on a 0.3–1.0 *M* linear sucrose gradient in a Beckman SW 65 Ti rotor at 65,000 rpm for 8 hr: total mRNA activity (●–●); casein mRNA activity (O–O); RNA concentration (Δ---Δ). The specific activities of fractions 9–15 are plotted on the insert. The values in parentheses represent the ratio of casein mRNA activity to total mRNA activity.

2.5% agarose gels in the presence of 6 *M* urea (Figure 6). The removal of 28S, 5S, and 4S RNAs, DNA, and some other mRNAs by Sepharose 4B chromatography is shown by a comparison of the first two gels. The removal of some additional 18S rRNA is then accomplished by dT-cellulose chromatography and sucrose gradient centrifugation. However, the resolving power of the gradients does not allow complete removal of 18S rRNA after a single centrifugation. Two minor RNA bands and a more intense RNA doublet in the 12 to 16S region of the gel are still evident in the first sucrose gradient peak fraction.

Sedimentation of the first sucrose gradient casein mRNA peak fraction on a second gradient, again performed after heat denaturation, allows the complete removal of contaminating 18S rRNA (Figure 7). A single peak of both absorbance at 260 nm and casein mRNA activity is now observed. However, when the casein mRNA to total mRNA activity ratio is plotted (insert, Figure 7) it is apparent that some additional noncasein mRNA activity is still present in the slower sedimenting region of the peak. Obviously, coexistence of absorbance at 260 nm and mRNA activity detected by specific immunoprecipitation alone is not sufficient proof of mRNA purity. Analysis of the peak activity fraction obtained after the second sucrose gradient centrifugation by electrophoresis on 2.5% agarose gels reveals the presence of only two major and one minor RNA band (Figure 8). When the RNA is extracted from the gels and its activity determined in the wheat germ assay both the casein mRNA activity and the total mRNA activity are found to comigrate with the major RNA bands at approximately 12 and 16 S. A small RNA absorbance peak at approximately 13 S

is also observed. Presumably these three RNA bands correspond to mRNAs able to code for the individual rat caseins of mol wt 25,000, 30,000, and 42,000. Unfortunately sufficient amounts of the purified casein mRNAs could not be obtained by elution from the gels by homogenization to allow a further product analysis. The improved resolution of the individual casein mRNAs by agarose gel electrophoresis compared to sucrose gradient centrifugation and the applicability of this technique to preparative gel electrophoresis (Rosen et al., 1975; Woo et al., 1975) should, however, permit the isolation of sufficient quantities of the individual rat casein mRNAs to allow their further characterization.

## Discussion

Since casein is synthesized in appreciable quantities during lactation and comprises almost 80% of the rat milk protein (Jenness, 1974) both the purification of the individual casein components and the detection of casein mRNA activity in total cell RNA extracts were greatly facilitated. Three principal kinds of rat casein were isolated from skim milk by isoelectric precipitation and DEAE chromatography, which have many of the same properties of the more thoroughly characterized $\alpha$, $\beta$, and $\kappa$ bovine and ewe caseins (McKenzie, 1971). Although during chromatography on DEAE-cellulose or acrylamide gel electrophoresis at pH 9.5 in the presence of 7 *M* urea (Feldman and Ceriani, 1970) rat casein has been shown to contain four components, two of these components were found to be of a similar apparent molecular weight by sodium dodecyl sulfate gel electrophoresis. These probably differ only in their extent of phosphorylation and/or carbohydrate content. However, such differences should not complicate the product analysis in the cell-free translation system, since purified ovalbumin mRNA, which also codes for a phosphorylated glycoprotein, has been shown to only direct the synthesis of a single molecular weight species in the wheat germ assay (Rosen et al., 1975). The lack of addition of carbohydrate in vitro may, however, slightly affect the mobility of the in vitro product as compared to the casein standards (Figure 3) or ovalbumin standard (Means et al., 1972). The one slice difference in the migration of the in vivo casein band 3 standard and the in vitro product synthesized in the wheat germ S-30 may also reflect the synthesis of a slightly longer precursor to casein band 3 in the cell-free system. Similar results have recently been reported for several other proteins synthesized in the wheat germ lysate (Boime et al., 1975; Kemper et al., 1974).

Purification of the individual rat caseins was found to be a necessary prerequisite for the generation of a specific antiserum. Purification methods which rely on isoelectric precipitation alone or utilize the rennin-$Ca^{2+}$ precipitation technique may result in casein preparations that are still contaminated with whey proteins or other nonphosphoproteins (Tan et al., 1972). Furthermore, rennin treatment may result in the limited proteolysis of casein. Rat casein proved to be a weak antigen and accordingly the generation of a high affinity casein antiserum was found to be extremely difficult. Removal of minor contaminants is, therefore, essential for the development of a definitive and specific immunoprecipitation assay. The quantitative determination of the activity of the individual rat casein mRNAs was, however, somewhat complicated with our antiserum. A similar difficulty in obtaining quantitative immunoprecipitation of rabbit casein has recently been reported (Houdebine and Gaye, 1975).

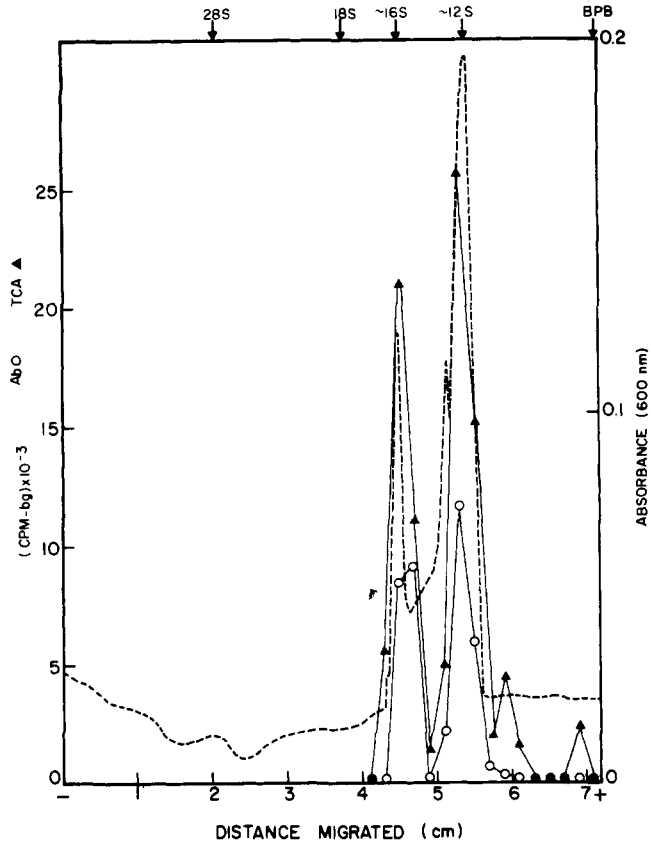


FIGURE 8: Analysis of purified casein mRNA by agarose gel electrophoresis. Approximately 11 $\mu$g of the peak fraction of casein mRNA activity obtained after the second sucrose gradient centrifugation was electrophoresed on 2.5% agarose gels in the presence of 6 *M* urea (see Experimental Procedures). Approximately 5 $\mu$g of the same RNA was also run on a parallel gel and the gel stained with 1% Methylene Blue. This gel was then scanned at 600 nm using a Gilford Model 2400 spectrophotometer, while the first gel was immediately sliced without staining and the RNA extracted as described: RNA absorbance (---); total mRNA activity (▲-▲); casein mRNA activity (O-O). The slightly uneven destaining rather than discrete RNA species accounts for the base-line variability.

With the development of a specific immunoprecipitation assay we were now able to purify two of the rat casein mRNAs by a combination of sizing and poly(A) adsorption techniques. Initially, rat casein mRNA was found to comprise a fairly large percentage (34–45%) of the total mRNA activity in a lactating mammary gland RNA extract. This was not unexpected since casein comprises a high percentage of rat milk proteins. Moreover, analogous results had previously been found for ovalbumin mRNA in the hen oviduct (Rosen et al., 1975), where ovalbumin comprises approximately 60% of the egg white protein. Thus, a whole cell RNA extract appears to be a suitable and easily obtainable starting material for casein mRNA purification. Casein mRNA activity can now be purified from DNA, 28S rRNA, 5S and 4S RNAs, and some additional mammary gland mRNAs, by chromatography on Sepharose 4B. In addition to the casein mRNA activity peak a second major peak of mRNA activity was found during Sepharose 4B chromatography (Figure 4). This peak consisted of mRNAs which synthesized proteins in the 15,000 to 23,000 mol wt range and may contain $\alpha$-lactalbumin mRNA activity. This large mRNA activity peak was absent in total nucleic acid extracts from chick liver, rat uterus, rat testes, and chick oviduct (Woo et al., 1974) and, therefore, appeared to be

unique to the mammary gland. The partial separation of casein mRNA activity from these other mRNAs was reflected in a 10% increase in the casein mRNA to total mRNA activity ratio.

Our observation that approximately 60% of rat casein mRNA, in either total RNA or Sepharose 4B purified RNA, was unable to bind to dT-cellulose is in agreement with previous results found for ewe $\alpha_s$-casein mRNA (Houdebine et al., 1974). Apparently, a large portion of both rat and ewe casein mRNAs lack poly(A) sequences of more than 20 adenosine residues and are unable to bind to poly(U)-Sepharose or dT-cellulose. Whether this is a result of aging of the casein mRNA (Sheiness and Darnell, 1973) or the lack of poly(A) addition to some of the initial casein mRNA transcripts (Milcarek et al., 1974) remains to be established. A somewhat smaller percentage (30%) of the total cell ovalbumin mRNA has also been found not to bind to dT-cellulose (Rosen et al., 1975). The comparison of casein mRNA activity during rat mammary gland development was, therefore, performed on total RNA extracts in order to avoid any such preferential losses of casein mRNA or total mRNA activity during partial purification.

The Sepharose 4B- and dT-cellulose purified RNA could be further purified by sucrose gradient centrifugation under denaturing conditions. The peak of casein mRNA activity was found to occur at 10.5 S. This result is consistent with the sedimentation value of 9 to 13 S that was found for total mRNA isolated by poly(U)-Sepharose chromatography from the ewe mammary gland (Gaye et al., 1973a,b). At this step in the purification scheme, almost 70% of the total mRNA activity present in the sucrose gradient peak was rat casein mRNA activity. This value is probably an underestimation for the following reasons: (1) the immunoprecipitation reaction was performed on the total wheat germ incubation mixture and may represent partially completed peptide chains not recognizable by our antiserum; (2) the immunoprecipitation reaction is not quantitative. Losses during immunoprecipitation can, however, be assessed with the use of high specific activity $^{14}C$- or $^{125}I$-labeled rat casein internal standards.[1] In addition the immunoprecipitation reaction can be performed only on released chains in the postmitochondrial supernatant. For example, in the case of ovalbumin mRNA these corrections resulted in a 20 to 30% increase in the amount of the mRNA activity detected as ovalbumin mRNA (Rosen et al., 1975). An analysis of the peptides synthesized in the cell-free system without the use of immunoprecipitation may also be used to assess the biological purity of the individual casein mRNAs. Unfortunately, following a second sucrose gradient centrifugation and analytical agarose gel electrophoresis, insufficient amounts of the individual casein mRNAs were eluted from the gel to perform this analysis. However, sufficient quantities of the 12S and 16S casein mRNAs to permit their detailed characterization have recently been obtained by a sequential combination, on a preparative scale, of the techniques described in this paper. Preparative agarose gel electrophoresis has been substituted for sucrose gradient centrifugation because of the superior resolution of comparably sized mRNAs (Woo et al., 1975) and an additional affinity chromatography step has been employed to remove contaminating rRNA fragments.

We have compared the levels of casein mRNA activity throughout rat mammary gland development. A selective induction of casein mRNA activity compared to total mRNA activity occurs during pregnancy leading to an increase in the casein mRNA to total mRNA ratio. This effect is coupled with a proliferation of alveolar cells and results in a 60-fold overall increase in casein mRNA activity from 5 days of pregnancy until early lactation. Following weaning there is a selective loss of casein mRNA and casein mRNA activity declined to barely detectable levels. These alterations in casein mRNA activity may be correlated with the serum levels of prolactin and placental lactogen which undergo marked changes in the rat during pregnancy, lactation, and after weaning (Shiu et al., 1973; Morishige et al., 1973). Both prolactin and placental lactogen have previously been shown to induce casein synthesis in several species (Turkington, 1968; Lockwood et al., 1966; Bourne and Bryant, 1974; Denamur and Delouis, 1972). Recently, there has been a direct demonstration of prolactin induction of casein mRNA activity in polysomes isolated from the pseudopregnant rabbit (Houdebine and Gaye, 1975).

The presence of appreciable amounts of biologically active casein mRNA during midpregnancy in the rat was unexpected since it is well known that appreciable casein secretion does not occur until parturition and the onset of lactation (Cowie and Tindall, 1971). It has been suggested by several investigators that the initiation of lactation is blocked by the high levels of ovarian steroids, specifically progesterone, that occur during pregnancy (Morishige et al., 1973; Davis et al., 1972; Denamur and Delouis, 1972). The availability of a sensitive assay for rat casein mRNA activity and procedures to purify the rat casein mRNAs with which to generate sensitive hybridization probes should now allow the direct assessment of the role of hormones in the transcription and translation of casein mRNA in both normal and malignant mammary tissue.

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[1] We have recently performed such a control experiment using a equal mixture by weight of the intact, $^{125}I$-labeled rat caseins as an internal standard. Under our assay conditions the efficiency of immunoprecipitation is routinely between 75 and 80%. Thus, a correction of approximately 20% should be added to all immunoprecipitation values.

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# Hybridization of Polymers of Antibiotic C-Nucleoside Phosphates, Poly(formycin phosphate) and Poly(laurusin phosphate)†

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ABSTRACT: The ability of complex formation of poly(formycin phosphate), poly(F), and poly(laurusin phosphate), poly(L), with the polymers of natural polynucleotides was examined mainly by mixing experiments in 0.1 *M* NaCl–0.05 *M* sodium cacodylate buffer (pH 7.0) at 2°. Poly(F) formed complexes with poly(U) and poly(I) in the ratio of 1:1 and 1:2, respectively. Poly(L) formed complexes with poly(A) in 2:1 ratio and poly(C) in 1:2 and 2:1 ratios in addition to a self-complex. Poly(F) and poly(L) also formed a 1:2 complex between them. Some of these complexes were assumed to contain novel types of base pairings using the 7-NH group. Thus it was concluded that poly(L) could form complexes with both, the oligomer of cycloadenylic acid ($\phi_{cn}$ −120°) and polymers of natural nucleotides ($\phi_{cn}$ 0°), showing flexibility of the torsion angle of the laurusin residue.

We have been investigating the effect of the torsion angle on the properties of oligo- and polynucleotides, mainly using cyclonucleoside derivatives, in which the torsion angle is fixed (Uesugi et al., 1972; Ikehara and Uesugi, 1972; Ikehara et al., 1974; Ikehara and Tezuka, 1973, 1974a,b). In this course, it is found that the oligomers of 8,2′-cycloadenylic acid form a left-handed helical structure (Uesugi et al., 1972; Ikehara and Uesugi, 1972, Ikehara et al., 1974), contrary to natural polynucleotides. The reason for this unusual conformation was ascribed to the value of the fixed torsion angle $\phi_{cn}$ (Donohue and Trueblood, 1960), about −120° (Tomita et al., 1972), which is in a syn-anti boundary region and is different from those of natural nucleosides possessing an anti conformation. Although these oligomers of 8,2′-cycloadenylic acid do not form complexes with the homopolynucleotides having natural nucleoside residues, the octamer of 8,2′-*S*-cycloadenosine 5′-monophosphate, $(pA^s)_8$,[1] is shown to form a left-handed helical complex

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[1] Abbreviations used are: $(pA^s)_8$, octamer of 8,2′-*S*-cycloadenosine 5′-monophosphate; poly(F), poly(formycin phosphate); poly(L), poly(laurusin phosphate).