Quantitation of Casein Messenger Ribonucleic Acid Sequences Using a Specific Complementary DNA Hybridization Probe†

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ABSTRACT: Two highly purified rat casein mRNA fractions were used as templates to synthesize complementary DNA (cDNA) hybridization probes using RNA-directed DNA polymerase isolated from avian myeloblastosis virus. Both of the probes selectively hybridized to RNA isolated from lactating mammary tissue, but not to poly(adenylic acid)-containing rat liver RNA. An analysis of the kinetics of hybridization of the cDNA derived from the 15S casein mRNA ($cDNA_{15S}$) or from the 12S casein mRNA ($cDNA_{12S}$) with their individual mRNA templates indicated that greater than 90% hybridization occurred over a R_0t range of one and one-half logs with $R_0t_{1/2}$ values of 0.0023 and 0.0032 mol s $l.^{-1}$, respectively. Compared with the total RNA isolated from lactating mammary tissue, these values represented a 166- and 245-fold purification, respectively, of these individual mRNA fractions. Using the 15S casein mRNA as a template, two probes of different lengths and specific activities were synthesized. The deoxyribonucleotide and mRNA concentrations and the temperature of incubation were optimized to obtain either a high specific activity cDNA probe, 330 nucleotides long, which represented approximately 25% of the mRNA or a lower specific activity preparation containing some complete cDNA copies, 1300 nucleotides in length. The T_m of the longer $cDNA_{15S}$–15S mRNA hybrid was 88.5 °C, while that of the short $cDNA_{15S}$–RNA hybrid was 82.5 °C. Following this initial characterization, the $cDNA_{15S}$ probe was utilized for three separate determinations: (1) Analysis of the sequence divergence between mouse and rat casein mRNAs. It was observed that the rate of hybridization of heterologous rat $cDNA_{15S}$–mouse casein mRNA was only 20% that of the homologous rat $cDNA_{15S}$–rat casein mRNA hybridization. The resulting heterologous hybrid displayed approximately 17% mismatching compared with the homologous hybrid. (2) Determination of the gene dosage for casein mRNA in normal and malignant mammary cells. In this study, an analysis of the kinetics of hybridization of the high specific activity $cDNA_{15S}$ probe with an excess of DNA isolated from lactating mammary tissue, carcinogen-induced mammary tumors, or rat liver indicated that casein mRNA was transcribed from the nonrepetitive DNA fraction of the rat genome. Furthermore, no major casein gene amplification or deletion was observed during tumor formation or the process of mammary differentiation. (3) Quantitation of casein mRNA sequences during normal mammary gland development. RNA excess hybridizations were performed using RNA extracted from either pregnant, lactating, or regressed rat mammary tissue. The concentration of casein mRNA molecules/alveolar cell was found to increase 12-fold from 5 days of pregnancy until 8 days of lactation and then declined to approximately 2% of the maximal level of 79 000 molecules/cell by 7 days after weaning. A coordinate increase was observed in casein mRNA sequences detected by cDNA hybridization and mRNA activity measured in a cell-free translation assay.

The hormone-dependent differentiation of the mammary gland during pregnancy and lactation is accompanied by a marked increase in the number of alveolar cells in the mammary fat pad (Munford, 1963). These specialized cells synthesize large quantities of the specific milk proteins, casein and α-lactalbumin, during lactation and then undergo regression following weaning (Turkington, 1971). Previous studies from our laboratory have demonstrated the selective induction of casein mRNA activity during pregnancy and lactation in the rat followed by a selective loss in activity during involution of the gland (Rosen et al., 1975a). However, the mechanism by which the polypeptide hormone, prolactin, acts to induce casein synthesis (Shiu and Friesen, 1976) and by which other hormones, such as progesterone and hydrocortisone, may modulate this response is not well understood.

Since casein mRNA activity was shown to comprise 50% of the total mRNA activity isolated from lactating rat mammary tissue, it was possible to purify two casein mRNA fractions essentially free of other contaminating mRNAs and rRNA (Rosen, 1976). These mRNAs have been utilized as templates for AMV^+ RNA-directed DNA polymerase to generate complementary DNA copies of the individual casein mRNA fractions. The enormous utility of such molecular hybridization probes is now well established. For example, complementary DNA copies of individual mRNAs have been used to establish the number of copies of a specific gene sequence in the cellular genome (Packman et al., 1972; Sullivan et al., 1973), to quantitate the number of copies of mRNA transcribed in vivo (Harris et al., 1975) or in vitro from isolated nuclei or chromatin templates (Gilmour and Paul, 1973), and to estimate the sequence divergence between different mRNAs (Leder et al., 1973). In addition, they have been extremely useful tools for the isolation of specific genes and mRNAs (Venetianer and Leder, 1974; Anderson and Schimke, 1976), for mRNA sequence determination (Marotta et al., 1974), and for the insertion of eukaryotic gene sequences into prokaryotic cells (Rabbitts, 1976). Conditions necessary for the synthesis of the full length DNA transcripts have been described recently (Efstratiadis et al., 1975; Monahan et al., 1976a). The synthesis of DNA complementary to the total rabbit milk proteins using *E. coli* DNA polymerase I has also been reported recently (Houdebine, 1976).

In the present study a specific probe for casein mRNA has

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been employed to answer the following questions: (1) Is casein mRNA transcribed from unique or repetitive sequences in the rat genome? (2) Does gene amplification or deletion occur during normal mammary differentiation or in carcinogen-induced mammary tumorigenesis? (3) Is the increase in casein mRNA activity observed during pregnancy and lactation correlated with an activation of previously "masked" mRNA sequences? Finally, the sequence divergence between rat and mouse casein mRNAs was also estimated using cDNA hybridization.

Experimental Procedures

Animals. The breeding of animals and isolation of mammary tissue have been previously described (Rosen et al., 1975a). Day zero of pregnancy is defined as the day which copulation plugs were found and the day of birth is designated as the first day of lactation. Mammary adenocarcinomas were induced in 50-day-old female Sprague-Dawley rats by a single intubation of 20 mg of DMBA.[1]

Nucleic Acid Isolation. DNA was isolated from rat liver, 10-day-lactating rat mammary tissue and DMBA-induced mammary carcinomas by extraction with chloroform–isoamyl alcohol–$NaDodSO_4$ as previously described (Rosen et al., 1973). A diphenylamine determination (Burton, 1968) and an optical density scan were performed on each DNA preparation. The DNA was then sheared at 16 000 psi in a French pressure cell press (Aminco) to yield fragments approximately 300–400 nucleotides in length (single-stranded). The isolation of total cellular RNA from pregnant, lactating, and regressed mammary tissue and rat liver by phenol–chloroform–$NaDodSO_4$ extraction at pH 8.0 has been described previously (Rosen et al., 1975a,b). Isolation of poly(A)-containing rat liver RNA was accomplished by (dT)-cellulose chromatography (Rosen et al., 1975a). The individual 15S and 12S casein mRNA fractions were purified from a total RNA extract of lactating tissue by chromatography on (dT)-cellulose and Sepharose 4B, followed by an additional (dT)-cellulose chromatographic procedure and preparative gel electrophoresis. Characterization of the 15S and 12S casein mRNA fractions was performed by both agarose–urea gel electrophoresis and product analysis in a wheat-germ, cell-free, translation assay (Rosen 1976).

Synthesis and Characterization of cDNA. Highly purified RNA-directed DNA polymerase isolated from avian myeloblastosis virus (preparation XL75-13, 40 573 units/mg) was kindly supplied by Dr. J. W. Beard through the auspices of Virus Cancer Program. The synthesis of ^3H-labeled cDNA copies of the 12S and 15S casein mRNAs was performed using two different protocols. The procedures were designed to generate either high specific activity cDNA transcripts of shorter length for use in DNA excess hybridization experiments or lower specific activity cDNA of a larger size which was more representative of the complete mRNA molecule for use in RNA excess hybridizations. The conditions for the synthesis of high specific activity cDNA were similar to those described by Monahan et al. (1976a). The reaction mixture contained in a final volume of 1.0 ml: 50 mM Tris-HCl, pH 8.3, 20 mM dithiothreitol, 6 mM $MgCl_2$, 200 μM dTTP, 5 μg/ml oligo-dT_{18-20}, 40 μg/ml actinomycin D, 1% ethanol, [5,8-^3H]dGTP (3.8 μM, 26.0 Ci/mmol), [8-^3H]dATP (14.3 μM, 7 Ci/mmol), [5-^3H]dCTP (4.5 μM, 22 Ci/mmol), 5 μg of purified 15S casein mRNA, and 124 units/ml of AMV RNA-directed DNA polymerase. The reaction was carried out at 46 °C for 15 min and the cDNA was isolated free of nucleotides and RNA as previously described (Harris et al., 1975). Under these conditions, the [^3H]cDNA had a specific radioactivity of 3.9×10^7 cpm/μg.

The following modifications were employed to generate cDNA of increased size which represented a more complete copy of the mRNA (see Figure 1). The reaction volume was adjusted to 0.1 ml and contained the same final concentration of each of the reactants except for the purified mRNA. The final casein mRNA concentration was increased from 5 to 50 μg/ml. The dGTP, dATP, and dCTP concentrations were also increased to 44, 54, and 45 μM, respectively, by the addition of 40 μM unlabeled deoxynucleotides. The conditions of incubation and isolation of the newly synthesized [^3H]cDNA were identical with those just described for the high specific activity cDNA. Using these modified conditions, the [^3H]cDNA had a calculated specific activity of 4.5×10^6 cpm/μg. The size of the cDNA product was determined by centrifugation on 8–18% (w/v) linear alkaline sucrose gradients (Monahan et al., 1975a) using sheared *E. coli* DNA (5.08 S) and ϕX174 DNA markers (Studier, 1965) as internal standards. Confirmation of the size of each cDNA preparation was obtained by electrophoresis on 99% formamide–polyacrylamide gels (Rosen et al., 1975a).

DNA and RNA Excess Hybridization. DNA excess hybridizations were carried out in a final volume of either 100 μl or 1.0 ml in tapered reaction vials (Regis Chemical Co.). A constant ratio of unlabeled DNA to [^3H]cDNA was maintained in each vial. Vials were incubated fully submerged in the water bath to prevent condensation and either the DNA concentration, the time of incubation, or Na^+ concentration was varied to obtain the desired C_0t values. Each reaction aliquot contained approximately 2000 cpm of [^3H]$cDNA_{15S}$, 350 nucleotides in length, and 740 μg of sheared DNA unless otherwise specified (see legend to Figure 6). Hybridization was performed in either 0.12 M phosphate buffer containing 1 mM Na_2EDTA at 62 °C or 0.48 M phosphate buffer containing 1 mM Na_2EDTA at 68 °C. Samples were initially heated at 100 °C for 5 min to ensure complete denaturation of the DNA. Following incubation the hybridization reaction was terminated by dilution into a final volume of either 3.0 (for 1.0-ml hybridization reactions) or 2.0 ml (for 100-μl hybridization reactions) at 4 °C, resulting in a final concentration of 0.14 M phosphate buffer. The extent of both hybridization and DNA renaturation was determined by hydroxylapatite chromatography (Rosen et al., 1973). Six 2.0-ml fractions of the 0.14 M (single-stranded) and 0.5 M phosphate (double-stranded) washes were collected. The A_{260} of each fraction was determined and 1.6 ml of the 0.14 M and 0.5 M fractions were counted in 9 ml of Aquasol (New England Nuclear) or 18 ml of Aquasol containing 1.5 ml of H_2O, respectively.

RNA excess hybridizations were performed in a final volume of 50 μl, containing 0.6 M NaCl, 0.01 M Hepes, pH 7.0, 0.002 M Na_2EDTA (Harris et al., 1975). Each hybridization reaction contained a minimum of 1500 cpm of[^3H]cDNA, varying amounts of mammary gland RNA (1 ng/ml to 2.25 mg/ml), and 400 μg/ml of hen oviduct RNA. Following heat denaturation for 30 s at 100 °C, incubations were performed

[1] Abbreviations used: AMV, avian myeloblastosis virus; $cDNA_{15S}$, DNA synthesized from the 15S casein mRNA; $cDNA_{12S}$, DNA synthesized from the 12S casein mRNA; $NaDodSO_4$, sodium dodecyl sulfate; DMBA, 7,12-dimethylbenz[*a*]anthracene; R_0t and C_0t, the concentration of RNA or DNA respectively, in mol $l.^{-1}$ times the time in seconds (mol s $l.^{-1}$); Na_2EDTA, disodium ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; poly(A), poly(adenylic acid); dT, deoxythymidine; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; RNP, ribonucleoprotein.

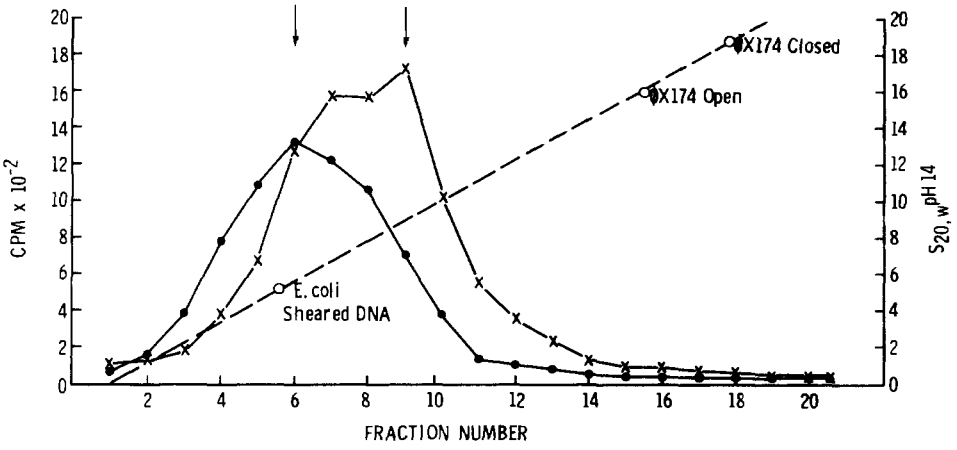

FIGURE 1: Size of $cDNA_{15S}$ determined by alkaline sucrose gradient centrifugation. Synthesis of $cDNA_{15S}$ was performed in a final volume of either 1.0 ml using a low substrate concentration (●) or in 0.1 ml using an increased substrate and mRNA concentration (X) as described under Experimental Procedures. The incubation was performed for 15 min at 46 °C and a portion of both samples centrifuged in a SW 40 rotor for 24 h at 38 000 rpm on 8–18% (w/v) linear sucrose gradients. The open circles designate the positions of the internal DNA standards and the arrows indicate the respective peak fractions of 5.05 $S_{20,w}^{pH14}$ and 9.5 $S_{20,w}^{pH14}$.

for 30 min to 16 h at 68 °C and the reactions terminated by freezing at −80 °C. The extent of hybridization was determined by S_1 nuclease digestion for 2 h at 37 °C in a buffer containing 0.2 M sodium acetate, pH 4.5, 0.475 NaCl, 2.5 mM $ZnCl_2$, and 150 μl of a DEAE-cellulose purified S_1 fraction (Harris et al., 1975) followed by Cl_3CCOOH precipitation and counting as described by Monahan et al. (1976a). Generally less than 4% of the [^{3}H]cDNA incubated in the absence of added mammary RNA was resistant to S_1 nuclease treatment. Two unincubated samples were included per experiment to assess the total Cl_3CCOOH-precipitable radioactivity prior to treatment with S_1.

The data from the hybridization experiments were expressed as the percent hybridization vs. the log equivalent C_0t (for DNA excess hybridization) and log equivalent R_0t (for RNA excess hybridization), where the rate has been adjusted to the standard conditions of 0.12 M phosphate (0.18 M Na^+) at 62 °C (Britten, 1969). The data were analyzed by a computer program which determined the best fit for an ideal pseudo-first-order (RNA excess) or second-order (DNA excess) curve to the respective hybridization data and also computed the apparent $R_0t_{1/2}$ and $C_0t_{1/2}$ values, respectively (Monahan et al., 1976b).

Fidelity of RNA-cDNA Hybrids. The T_m determination of mRNA-cDNA hybrids was performed using S_1 nuclease, rather than hydroxylapatite in order to minimize the effect of the poly(A)-poly(dT) interaction present in all mRNA-cDNA hybrids. RNA excess hybridizations were allowed to proceed to completion and then 50-μl aliquots of the reaction containing at least 1000 cpm of hybridized [^{3}H]cDNA were diluted to 0.2 M Na^+ by the addition of 100 μl of H_2O. Each sample was then allowed to equilibrate at the designated temperature for 5 min and finally rapidly frozen at −80 °C in a dry ice–acetone bath. The amount of remaining hybrid was then determined by S_1 nuclease treatment as previously described. The data are expressed as the cumulative percentage of the total hybridized [^{3}H]cDNA that was sensitive to S_1 treatment as a function of the temperature.

Results

Several recent reports have suggested that full-length complementary DNA transcripts of individual mRNAs can be synthesized using AMV RNA-directed DNA polymerase by adjusting both the deoxynucleotide triphosphate substrate concentration and the time and temperature of incubation (Efstratiadis et al., 1975; Monahan et al., 1976a). In addition to these components the final size of the cDNA transcript of the 15S casein mRNA was found to be dependent upon the relative mRNA and enzyme concentrations (Figure 1). When the tritiated deoxynucleotide triphosphate substrate concentrations were maintained at approximately 4 to 14 μM and the mRNA concentration was 5 μg/ml, a 350 nucleotide long $cDNA_{15S}$ (5.5 $S_{20,w}^{pH14}$) was synthesized with a specific activity of 3.9×10^7 cpm/μg. A similarly sized cDNA copy was also observed under comparable reaction conditions using the 12S casein mRNA as a template (data not shown). In both cases a symmetrical peak was observed during alkaline sucrose gradient centrifugation (Figure 1), and further analysis of the rapidly sedimenting cDNA fractions did not reveal any full length transcripts.

Raising the substrate concentration of the three radioactive deoxynucleotide triphosphates to a minimum of 40 μM under these same incubation conditions was not sufficient to allow the synthesis of a full length $cDNA_{15S}$ transcript. However, if the mRNA concentration was increased to 50 μg/ml concomitantly with the increase in substrate concentration, a substantially larger $cDNA_{15S}$ was observed (Figure 1). When the rapidly sedimenting cDNA fractions synthesized under these conditions (approximately 9.5 $S_{20,w}^{pH14}$) were analyzed by electrophoresis on acrylamide gels containing 99% formamide, the estimated size of the cDNA transcript was 1300 nucleotides representing essentially a complete copy of the 15S casein mRNA (data not shown). Increasing the mRNA concentration to greater than 50 μg/ml was accomplished by a tenfold reduction in the reaction volume, while the concentration of all other components was held constant (Table I). Under these conditions only a fourfold decrease in the efficiency of cDNA synthesis was observed, even though the amount of AMV polymerase was reduced tenfold. Thus, both the enzyme and mRNA concentrations appear to be critical variables in the reaction. Both conservation of the limiting amounts of purified mRNA and an increase in the size of the cDNA transcript resulted from these modifications.

Characterization of the individual $cDNA_{15S}$ and $cDNA_{12S}$ probes included an analysis of the kinetics of hybridization with their respective mRNA templates, with a total RNA extract from a lactating gland and with liver poly(A)-RNA. These experiments were initially performed with high specific activity

TABLE I: Effects of mRNA and AMV Polymerase Concentrations on $cDNA_{15S}$ Synthesis.

	Reaction Vol (μl)	mRNA (μg)	AMV Polymerase (Units)	cDNA Synthesized (ng)	Efficiency of Synthesis (%)
(A)	1000	4.85	12.4	380	7.8
(B)	100	6.76	12.4	128	1.9
(C)	100	6.15	12.4	122	2.0

[a] The concentrations of all other components are identical with those described in Experimental Procedures for the complete $cDNA_{15S}$ synthesis. The reaction was incubated for 15 min at 46 °C.

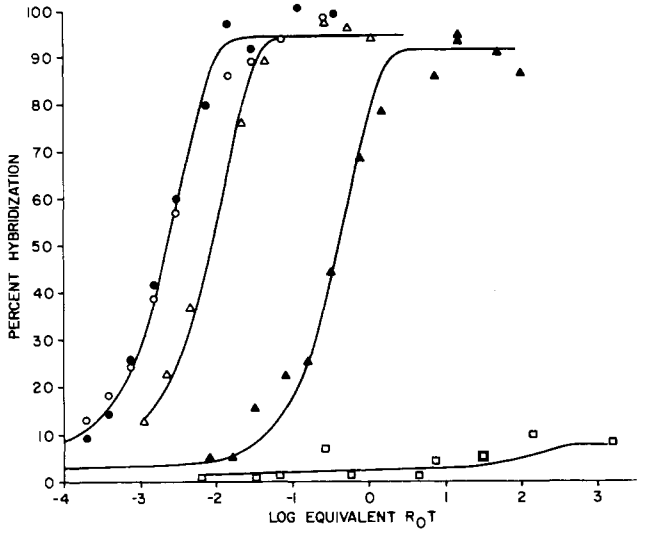

FIGURE 2: Specificity of the $cDNA_{15S}$ probe. RNA excess hybridizations were performed with a purified 15S rat casein mRNA fraction (●, ○), purified 12S rat casein mRNA (△), a total lactating RNA extract (▲), and rat liver poly(A)-containing RNA (□), with the 350 nucleotide long $cDNA_{15S}$ transcript. The open circles designate the use of an unfractionated $cDNA_{15S}$ preparation in the hybridization reaction with 15S mRNA, while the closed circles designate the use of a fractionated probe (fractions 6–10, solid circles of Figure 1) obtained after alkaline sucrose gradient centrifugation. Hybridization conditions and assay procedures are described in Experimental Procedures.

cDNAs approximately 350 nucleotides long. Greater than 90% hybridization of the cDNA with purified 15S casein mRNA occurred over a R_0t range of one and one-half logs with an apparent $R_0t_{1/2}$ of 0.0023 mol s $l.^{-1}$ (Figure 2). An improved fit with an ideal pseudo-first-order hybridization curve was also observed when the shortest cDNA transcripts (<100 nucleotides) were removed by prior alkaline gradient centrifugation (Figure 2). The rate of hybridization of the $cDNA_{15S}$ with the total lactating RNA preparation was 166 times slower ($R_0t_{1/2}$ = 0.38 mol s $l.^{-1}$) than with the purified mRNA template. This was in close agreement with the previous estimation of mRNA purity obtained by translation in the wheat-germ, cell-free system (Rosen, 1976). The specificity of the $cDNA_{15S}$ was demonstrated by its failure to hybridize significantly with poly(A)-containing RNA isolated from rat liver even at a R_0t value of 1000 mol s $l.^{-1}$. The $cDNA_{15S}$ was also utilized to determine the extent of homology or contamination of the purified 12S mRNA fraction with 15S casein mRNA sequences. The rate of the cross-hybridization reaction ($cDNA_{15S}$ vs. 12S mRNA) was almost threefold slower than

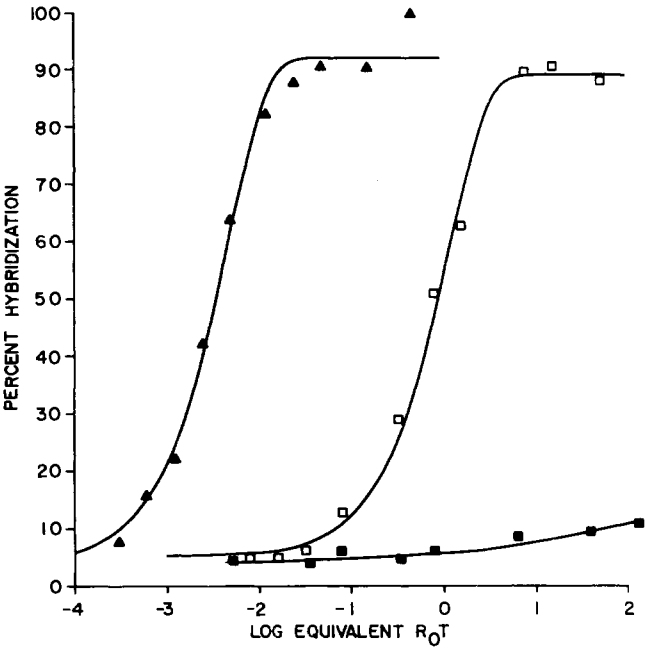

FIGURE 3: Specificity of the $cDNA_{12S}$ probe. RNA excess hybridizations were performed with purified rat 12S mRNA (▲), a total lactating RNA preparation (□), and a rat liver poly(A)-containing RNA extract (■) using a 350 long $cDNA_{12S}$ transcript.

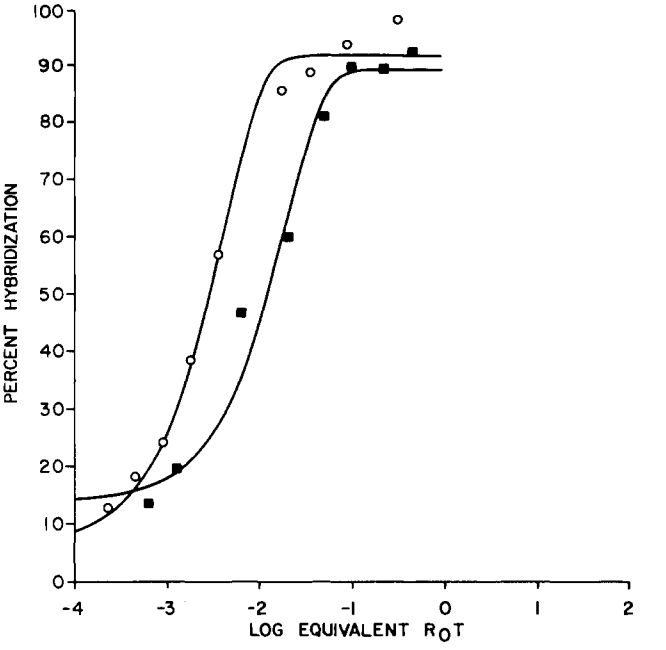

FIGURE 4: Sequence homology between rat and mouse 15S casein mRNAs. The kinetics of hybridization of rat 15S casein mRNA (○) and mouse 15S casein mRNA (■) with the 350 nucleotide long $cDNA_{15S}$ were studied as described under Experimental Procedures.

that of the homologous reaction, i.e., $R_0t_{1/2}$ = 0.0081 vs. 0.0023 mol s $l.^{-1}$, but both reactions went to greater than 90% completion.

Similar hybridization experiments were also performed with $cDNA_{12S}$ (Figure 3). Again the hybridization occurred with the expected pseudo-first-order kinetics and the extent of hybridization was greater than 90%. However, in this case the rate of hybridization of $cDNA_{12S}$ with its template mRNA was 245 times faster than with the initial lactating RNA extract

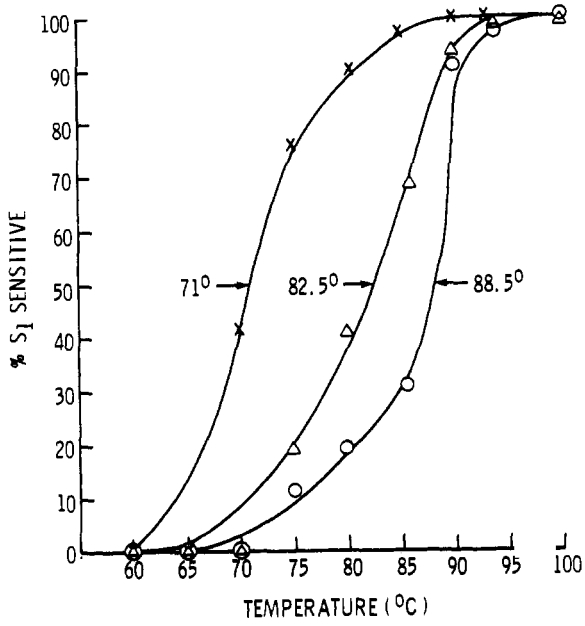

FIGURE 5: Thermal stability of mRNA-$cDNA_{15S}$ hybrids. The thermal stability of the hybrids in 0.2 M Na^+ was assessed by treatment with S_1 nuclease following 5-min equilibration at the designated temperatures. The arrows indicate the respective T_m's of a 350 nucleotide long $cDNA_{15S}$-rat 15S mRNA hybrid (△), a 350 nucleotide long $cDNA_{15S}$-mouse 15S mRNA hybrid (X), and a long $cDNA_{15S}$-rat 15S mRNA hybrid (○).

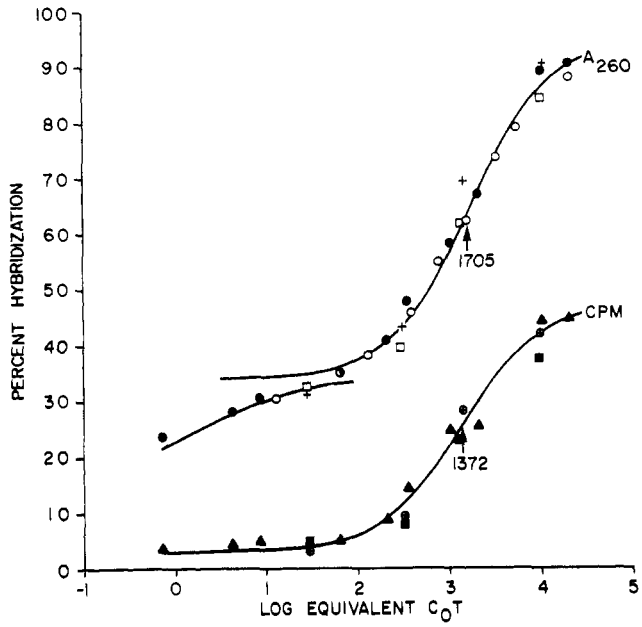

FIGURE 6: Determination of the casein gene dosage in rat DNA. DNA excess hybridizations were performed as described using 737 μg of rat liver DNA (●, ○) and 5.08×10^{-5} μg of [^{3}H]$cDNA_{15S}$ (▲, specific activity = 3.9×10^6 cpm/μg) in a final volume of either 0.1 or 1.0 ml. The open and closed circles represent the results of two separate experiments. Comparable experiments were also performed with either 476 μg of DNA isolated from lactating mammary tissue (+, ⊕) or 662 μg of DNA isolated from DMBA-induced mammary carcinomas (□, ■) using the [^{3}H]$cDNA_{15S}$ at a similar complementary sequence ratio to that used in the rat liver DNA experiment. The extent of hybridization was analyzed by hydroxylapatite chromatography as described. The A_{260} curve represents the renaturation of the respective nonradioactive DNAs and the cpm curve designates the hybridization of the [^{3}H]$cDNA_{15S}$.

($R_0t_{1/2}$ = 0.0032 vs. 0.78 mol s l.$^{-1}$). This value was considerably greater than the expected increase predicted from the cell-free translation assay (Rosen, 1976). This cDNA was also a specific probe for casein mRNA sequences and did not hybridize significantly to rat liver poly(A)-RNA.

In order to characterize further the rat $cDNA_{15S}$, the kinetics of hybridization of the rat $cDNA_{15S}$ with purified mouse 15S casein mRNA were studied (Figure 4). Similar types of experiments performed using hemoglobin mRNAs isolated from several species and their respective cDNA transcripts have illustrated the sensitivity to this approach for detecting sequence divergence between different mRNAs (Leder et al., 1973; Gummerson and Williamson, 1974). The rate of hybridization of the rat $cDNA_{15S}$ with mouse 15S mRNA was approximately one-fifth as fast as the homologous rat $cDNA_{15S}$-mRNA hybridization. A $R_0t_{1/2}$ value of 0.0124 mol s l.$^{-1}$ was obtained. Both reactions again went to at least 90% completion.

The fidelity of hybridization was then ascertained by determining the thermal stability of the resulting cDNA-mRNA hybrids. The homologous hybrid formed between the 350 nucleotide long $cDNA_{15S}$ and 15S casein mRNA displayed a sharp melting profile with a T_m of 82.5 °C (Figure 5). The T_m of the heterologous $cDNA_{15S}$-mouse 15S mRNA hybrid was reduced by 11.5 °C, indicating approximately 17% mismatching compared with the homologous hybrid (Kohne et al., 1971). The thermal stability of a hybrid formed between the more complete $cDNA_{15S}$ transcript and rat 15S casein mRNA was also determined. An increase in the T_m of 6 °C was observed with the longer $cDNA_{15S}$-15S mRNA hybrid. A similar high T_m was observed for an ovalbumin mRNA-long cDNA hybrid (Monahan et al., 1976a). The presence of a lower melting component, which consisted of only 10 to 20% of the $cDNA_{15S}$, was probably due to some short cDNA transcripts in the unfractionated cDNA preparation.

Having established both the specificity and fidelity of hybridization of the $cDNA_{15S}$ probe, experiments were next performed to determine the gene dosage in rat DNA for the 15S casein mRNA. DNA was isolated from rat liver, lactating rat mammary tissue, and DMBA-induced rat mammary carcinomas. A large mass excess of DNA from each of these sources was then sheared to a mean size of 350 nucleotides and incubated with the high specific activity $cDNA_{15S}$ of similar size. The kinetics of reassociation of rat DNA and the rate of [^{3}H]cDNA hybridization were analyzed by chromatography on hydroxylapatite (Figure 6). The observed $C_0t_{1/2}$ of rat unique sequence DNA of 1705 mol s l.$^{-1}$ was similar to previously published values (Holmes and Bonner, 1974). The rate of hybridization of the [^{3}H]cDNA indicated that the 15S casein mRNA was transcribed from nonrepetitive DNA sequences. The observed $C_0t_{1/2}$ of 1372 of the [^{3}H]cDNA was similar to that determined for the rat unique DNA. The extent of hybridization was approximately 45% and was a reflection of the lack of a true excess of the complementary sequence for the casein cDNA even at a mass ratio of DNA to [^{3}H]cDNA of 1.45×10^7. The inability to achieve a complementary sequence excess for unique gene sequences is a limitation of these experiments utilizing mammalian DNA (Ross et al., 1974). No significant differences were observed in the hybridization kinetics of the [^{3}H]$cDNA_{15S}$ with DNA isolated from either normal rat liver, differentiated mammary tissue, or mammary adenocarcinomas (Figure 6). This suggested that major gene amplification or deletion could not account for the alterations in casein mRNA activity observed during normal development and tumorigenesis (Rosen et al., 1975a).

TABLE II: Coordinate Increase in Casein mRNA Sequences Detected by cDNA Hybridization and Casein mRNA Translation Activity during Rat Mammary Development.

Stage	$R_0t_{1/2}$[b]	Fraction Casein mRNA (%)	Casein mRNA (cpm/μg)	RNA/DNA[c]	%[d] Alveolar Cells	No. of Molecules[e] of Casein mRNA per Alveolar Cell
8-day lactating	0.48	0.52 (38×)	22 500 (45×)[a]	2.50	75	79 000
20-day pregnant	0.93	0.27 (20×)	11 500 (23×)	1.96	45	55 000
10-day pregnant	1.9	0.13 (9×)	3 900 (8×)	1.57	25	37 000
5-day pregnant	9.3	0.027 (2×)	1 100 (2×)	0.53	10	6 000
7-day postweaning	17.9	0.014	500			

[a] Fold increase compared with 7-day postweaned. [b] $R_0t_{1/2}$ of 15S casein mRNA = 2.5×10^{-3}. [c] From Chatterton et al. (1975), Russell and McVicker (1972), and Liu and Davis (1967). A value of 6.5 pg of DNA was used per alveolar cell (Sober, 1970). [d] From Munford (1963). [e] The total complexity of the 15S casein mRNA doublet assuming the presence of 2 mRNAs of approximately 400 000 and 450 000 equals 850 000.

In order to quantitate the levels of casein mRNA during the normal development of the rat mammary gland, the $cDNA_{15S}$ probe was hybridized with an excess of RNA isolated from mammary tissue at different stages of pregnancy, during lactation, and following regression of the gland after weaning (Figure 7). A series of parallel hybridization curves was generated which displayed progressively faster rates of hybridization from 7-day postweaning to 8 days of lactation. Casein mRNA sequences represented 0.52% of the total cellular RNA in the 8-day lactating mammary tissue, a 19-fold increase over the amount present at 5 days of pregnancy (Table II). Following regression of the gland, the level of casein mRNA sequences decreased markedly until they comprised only 0.014% of the total RNA. The amount of 15S casein mRNA sequences in the total tissue RNA population was also expressed as the number of molecules of casein mRNA per alveolar cell (Table II). This calculation requires an estimation of the percentage of alveolar cells in the mammary gland at each stage of development (Munford, 1963), the complexity of the 15S casein mRNA (Rosen, 1976), the RNA and DNA content of the mammary gland at each developmental period (Russell and McVicker, 1972; Chatterton et al., 1975; Liu and Davis, 1967), and the DNA content per cell (Sober, 1970). A 12-fold increase in the number of molecules of casein mRNA per alveolar cell was observed between 5 days of pregnancy and 8 days of lactation reaching a maximal level of 79 000 molecules per cell. Thus, the observed increase in the fraction of casein mRNA in the total RNA extract represents both the induction of casein mRNA per alveolar cell and an increase in the number of alveolar cells with the capacity to synthesize casein mRNA.

Since the cDNA hybridization assay will not discriminate between total casein mRNA sequences and functional casein mRNA activity, these same RNA extracts were assayed in the wheat-germ, cell-free, translation system (Table II). A coordinate increase in both casein mRNA sequence concentration and translation activity was observed in these RNA preparations. The 5-day-pregnant RNA samples contained a twofold greater sequence concentration and translation activity than the RNA isolated from the 7-day postweaned, mammary tissue. By 8 days of lactation the casein mRNA sequence concentration had increased 38-fold, and the mRNA activity displayed a corresponding 45-fold increase compared with the regressed mammary gland. These results suggested that appreciable amounts of biologically inactive mRNA were not being synthesized during early and mid pregnancy and later activated at parturition or during lactation.

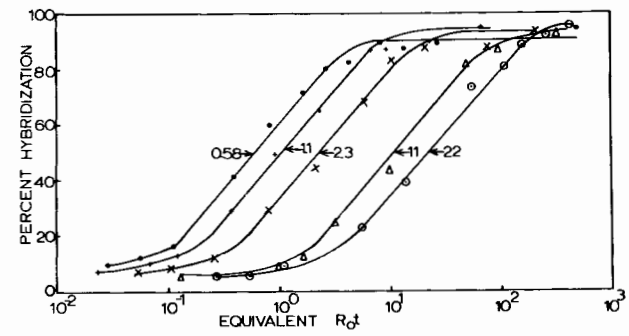

FIGURE 7: Quantitation of casein mRNA sequences during pregnancy, lactation, and regression of the rat mammary gland following weaning. RNA excess hybridizations were performed using the 350 nucleotide long $cDNA_{15S}$ and RNA obtained from mammary tissue after 8 days of lactation (●), 20 days of pregnancy (+), 10 days of pregnancy (X), 5 days of pregnancy (△), or 7 days after weaning (⊙). The arrows designate the respective $R_0t_{1/2}$ values for each hybridization.

Discussion

The conditions necessary to synthesize either a high specific activity cDNA probe for use in gene dosage experiments or a longer cDNA probe that was more representative of the complete 15S casein mRNA were essentially similar to those previously described for DNAs complementary to ovalbumin mRNA (Monahan et al., 1976a) and hemoglobin mRNA (Efstratiadis et al., 1975). However, it was necessary to maintain a high mRNA concentration of at least 50 μg/ml in order to generate a cDNA transcript that represented a full-length copy of the 15S casein mRNA. Even under these conditions, a spectrum of cDNA lengths was observed ranging between 350 and 1300 nucleotides. Conceivably the proportion of full-length transcripts may be increased further by raising the deoxynucleotide triphosphate concentration and optimizing the enzyme and mRNA concentrations.[2] Alternatively the

[2] Added in Proof. We have recently performed such experiments raising the dATP, dCTP, and dTTP concentrations to 400 μM final concentrations and using [^{3}H]dGTP (4.3 Ci/mmol) at 116 μM. The Mg^{2+} concentration was raised to 9 mM to compensate for the increased deoxynucleotide triphosphate concentrations and the reaction was performed in a final volume of 1.0 ml containing 62 μg of purified 15S casein mRNA. Under these conditions, 2500 ng of cDNA (specific activity 3.1×10^6 cpm/μg) was synthesized at an efficiency of 4%. The proportion of full-length cDNA increased under these conditions from 20 to 60% of the total product synthesized, as analyzed by alkaline sucrose gradient centrifugation. This full-length cDNA protected 100% of an ^{125}I-labeled 15S casein mRNA from nuclease digestion at a cDNA/mRNA ratio of 2 (Rosen and Barker, unpublished observations).

complete cDNA transcript may be obtained by fractionation of the total reaction product on alkaline sucrose gradients.

A comparison of the thermal stability of the hybrids formed between the 350 nucleotide $cDNA_{15S}$ or the 1300 nucleotide $cDNA_{15S}$ with the 15S rat casein mRNA template indicated a 6 °C increase in the T_m of the longer cDNA–mRNA hybrid. This increase can be explained not only by the increased length of the hybrids, but also by a higher GC content of the 3′ end of the $cDNA_{15S}$ and accordingly the 5′ end of the 15S casein mRNA, due to the presence of a 3′-terminal poly(A) sequence in the mRNA. The mRNA poly(A) tail–cDNA poly(dT) interaction should comprise approximately 13% of the 350 nucleotide long cDNA hybrid but only 3% of the 1300 nucleotide transcript. However, the increase in T_m may also reflect the high concentration of guanine-rich sequences at the 5′ end of the mRNA coding for the glutamic acid and serine residues known to occur at the N-terminal regions of the α_s and β bovine caseins (Taborsky, 1974). The high T_m of 88.5 °C observed for the longer $cDNA_{15S}$–mRNA hybrid is consistent with the values observed for other well-matched cDNA–mRNA duplexes (Schechter, 1975; Monahan et al., 1976a). Determination of the T_m using S_1 nuclease treatment in 0.2 M Na^+ would be expected to yield a slightly lower value than that obtained by thermal denaturation studies performed by hydroxylapatite chromatography in 0.14 M phosphate buffer because of the increased stability of such hybrids on hydroxylapatite (Martinson, 1973).

In addition to the fidelity of base pairing observed with the $cDNA_{15S}$ and $cDNA_{12S}$ probes, the specificity of hybridization was demonstrated by their failure to hybridize significantly with rat liver poly(A)-containing RNA. A slight increase in hybridization of approximately 5% routinely observed in these experiments at R_0t values greater than 10 could not be explained by an increased S_1 background when large quantities of RNA were employed or by any potential DNA contamination in the RNA preparation. Since total cell poly(A)-RNA extracts were used in these control experiments, it is conceivable that this low level of hybridization may represent a low level of casein mRNA sequences present in the poly(A)-RNA of nonmammary tissues. A similar observation has recently been made for globin sequences in nonerythroid cells (Humphries et al., 1976). The significance of this result will require further characterization of casein-specific cDNAs, as well as the nonmammary RNA–cDNA hybrids.

Characterization of the $cDNA_{15S}$ and $cDNA_{12S}$ probes also provided additional support for the purity of the individual rat casein mRNA fractions. Both the kinetics and selectivity of hybridization of the $cDNA_{15S}$ are consistent with the absence of any major contaminants in the purified 15S mRNA fraction. An excellent correlation was observed between the 178-fold purification estimated by translation activity in the wheat-germ system (Rosen, 1976) and the 166-fold increase in the rate of hybridization of the 15S mRNA compared with the lactating RNA extract. In addition, the $R_0t_{1/2}$ value of the 15S casein mRNA with the $cDNA_{15S}$ determined by hybridization was 2.3 to 2.5 $\times 10^{-3}$ mol s $l.^{-1}$, consistent with the purity of the mRNA. However, without the use of internal mRNA and cDNA standards of known sequence complexities in these hybridization experiments, it was difficult to estimate the absolute purity of the mRNA by such a kinetic analysis. For example, the equivalent $R_0t_{1/2}$ (0.18 M Na^+ at 62 °C) of purified ovalbumin mRNA (complexity, 650 000) has been reported to range from 4.6 $\times 10^{-3}$ mol s $l.^{-1}$ using hybridization conditions similar to those employed in this manuscript (Monahan et al., 1976a) to 2.6 $\times 10^{-3}$ mol s $l.^{-1}$ (Levy and McCarthy, 1975; Axel et al., 1976) using different incubation conditions and assay methods. Furthermore, in order to determine the theoretical $R_0t_{1/2}$ value for a purified 15S casein mRNA fraction, an accurate assessment of its sequence complexity was necessary. Since the mRNA fraction appeared as a doublet during agarose–urea gel electrophoresis and specified two proteins of different sizes in the wheat-germ translation assay, a total complexity of approximately 850 000 was assumed for this fraction. This complexity was used to calculate the number of molecules of casein mRNA per alveolar cell. This value assumes the presence of equal amounts of two casein mRNAs of molecular weights 400 000 and 450 000. However, until these two mRNAs can be separated, their individual complexities and theoretical $R_0t_{1/2}$ values cannot be more accurately assessed.

Although the 12S casein mRNA was estimated to be purified 95-fold compared with the initial lactating RNA extract based on immunoprecipitation data obtained in the wheat-germ translation assay (Rosen, 1976), this fraction represented greater than a 200-fold purification when the kinetics of $cDNA_{12S}$ hybridization were analyzed. Thus, in this case there is a considerable discrepancy between these two methods, which may reflect the inability of the anticasein IgG fraction to quantitatively precipitate the cell-free product. The $cDNA_{12S}$ probe also failed to hybridize to any appreciable extent with rat liver poly(A)-containing RNA. However, hybridization analysis did reveal approximately a 35% cross-contamination of the 12S mRNA with a fragment of the 15S mRNA. Both the homologous hybridization reaction of the $cDNA_{15S}$ with the 15S mRNA and the cross-hybridization with the 12S mRNA proceeded to greater than 90% completion and the T_m's of both hybrids were identical (88.5 °C). Thus, the rate and extent of cross-hybridization probably do not reflect sequence homology between the two mRNAs, but rather contamination of the smaller 12S mRNA with a fragment of the larger 15S mRNA. Removal of the contaminating 15S mRNA sequences potentially may be accomplished by passage of the 12S mRNA fraction through a $cDNA_{15S}$ affinity column and isolation of the nonhybridized 12S mRNA. This type of experimental approach may be required before the individual casein cDNA probes can be utilized for definitive studies of the coordinate regulation of the individual casein mRNAs. Analogous results have been reported for α and β rabbit hemoglobin mRNAs (Velez et al., 1975). Analysis of the mRNA–cDNA hybridization reactions indicated an enrichment in either α and β mRNA sequences in the respective mRNA fractions, but significant cross-hybridization also occurred.

The extreme sensitivity of the cDNA hybridization assay, therefore, permitted the detection of sequence contamination which was not apparent using the more conventional mRNA characterization techniques. The use of sizing techniques alone for the purification of specific mRNAs may result in the co-purification of such poly(A)-containing mRNA fragments, especially when a larger mRNA comprises a significant proportion of the total mRNA population.

The sequence divergence observed for mouse and rat 15S casein mRNAs was slightly greater than the limited differences previously reported for rat and mouse hemoglobin mRNAs (Gummerson and Williamson, 1974). While mouse globin cDNA hybridized to both mouse and rat globin mRNA to 80% completion, the rate of the heterologous hybridization was one-third that of the homologous reaction and only a 2 °C decrease in the T_m of the respective hybrids was reported (76 vs. 74 °C). An 11 °C decrease in the T_m of the mouse–rat ca-

sein mRNA-$cDNA_{15S}$ hybrid was observed reflecting 17% mismatching (Kohne et al., 1971) and the rate of hybridization was 20% of the rat $cDNA_{15S}$-mRNA reaction. However, direct comparison of these results is difficult because the rate of the globin mRNA-cDNA hybridizations was analyzed by hydroxylapatite chromatography and the T_m's observed were considerably lower than expected for well-matched globin cDNA-mRNA hybrids. A limited homology between rat and mouse caseins was previously suggested by a weak cross-reactivity between an anti-rat casein IgG fraction and mouse casein (Rosen, unpublished observations). However, the different apparent molecular weights of the mouse and rat caseins indicate the presence of some degree of divergence between these proteins (Rosen, 1976). Unfortunately amino acid sequence data are not available to permit a direct comparison of the protein and mRNA interspecific homologies. The specificity of the $cDNA_{15S}$ probe was, however, further supported by this analysis which indicated the ability of the $cDNA_{15S}$ to discriminate among very similar nucleotide sequences.

Evidence for the transcription of casein mRNA from nonrepetitive DNA sequences was obtained by the analysis of the rate of hybridization of the [^{3}H]$cDNA_{15S}$ with an excess of rat DNA. This type of analysis has been described in detail by Ross et al. (1974). Gene dosage experiments have now been performed for a number of specific mRNAs (Packman et al., 1972; Sullivan et al., 1973; Rosen et al., 1974; Harrison et al., 1974) and have demonstrated that a single gene copy specifies these mRNAs. In several instances the number of gene sequences have been compared in both total genomic DNA and DNA isolated from a specific, differentiated tissue. No major gene amplification or deletion has been reported. An identical observation has been made for the gene complement specifying the 15S casein mRNA. In addition, an analysis of DNA isolated from DMBA-induced mammary adenocarcinomas indicated that, during the process of neoplastic transformation, the structural gene for the 15S casein mRNA is also conserved. This technique cannot at present distinguish between the presence of one gene copy specifying both of the 15S casein mRNAs or an individual gene complement for each of the mRNAs. Interestingly, genetic evidence has suggested that the individual α and β bovine casein genes may be closely linked (Taborsky, 1974).

The absence of casein gene amplification suggests that either selective transcription of casein mRNA or posttranscriptional regulation of casein mRNA levels must account for the changes previously observed in casein mRNA activity during rat mammary development (Rosen et al., 1975a). The availability of a specific $cDNA_{15S}$ probe allowed a direct quantitation of the number of casein mRNA molecules per alveolar cell. The large number of molecules of casein mRNA (79 000 per cell) observed in the lactating RNA preparation is consistent with previous results. Casein mRNA activity was previously shown to represent approximately 50% of the total mRNA activity in the lactating mammary gland (Rosen et al., 1975a). This result was supported by the observation that casein mRNA comprised 0.52% of the total cellular RNA as determined by direct hybridization analysis and the total amount of mRNA has been estimated to range between 1 and 1.5% of the cellular RNA. Thus, in highly specialized tissues which are producing large amounts of a given protein, it is not unusual to observe mRNA levels as high as 80 000 to 100 000 specific mRNA molecules per cell. For example, the levels of casein mRNA observed in the lactating mammary gland were comparable to the number of ovalbumin mRNA molecules determined per tubular gland cell in the fully stimulated hen oviduct (Harris et al., 1975; McKnight et al., 1975).

A coordinate increase in both casein sequences and casein mRNA activity occurred during the development of the rat mammary gland from pregnancy to lactation. These data suggested that the regulation of casein synthesis during lactation most likely does not involve the activation of previously inactive mRNA. However, these results cannot rule out the possibility that casein mRNA sequences are sequestered in the nucleus or as inactive mRNP particles in the cytoplasm during pregnancy. Recent data from our laboratory has indicated that casein mRNA is, in fact, associated with mammary polysomes isolated from the pregnant rat and that these polysomes are capable of synthesizing casein in vitro (Rosen and Comstock, 1976). The availability of a sensitive and selective probe for casein mRNA sequences should now permit a more direct examination of the regulation of casein mRNA transcription and casein synthesis and the role of hormones in these processes. In addition, the $cDNA_{15S}$ may be used to screen mammary carcinomas for the presence of casein mRNA sequences (Socher and Rosen, 1976) and may, therefore, be a useful molecular marker for hormone-dependent breast cancer.

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A Slow Interconversion between Active and Inactive States of the (Na-K)ATPase†

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ABSTRACT: We have examined slow changes in the rate of ATP hydrolysis for purified dog kidney Na^+ and K^+ stimulated adenosine triphosphatase [(Na-K)ATPase] at various concentrations of free Mg^{2+}, Mg-ATP, K^+, and Na^+. The effect of these ligands on the rate of ATP hydrolysis is explained by a rapid binding step determining the initial rate of turnover followed by a slow conformational change. Inactivation of enzyme stored in the presence of ethylenediaminetetraacetic acid occurs upon adding free Mg^{2+}, Mg-ATP, and K^+; reactivation may be achieved if the concentration of these ligands is reduced. Because of the slow conformational change, the affinities for ligands affecting inactivation are time dependent. A model is presented to explain the effects of free Mg^{2+} and Mg-ATP on (Na-K)ATPase activity.

Studies of the Na^+ and K^+ stimulated ATPase [(Na-K)-ATPase][1] have provided important information about the mechanism of ion transport by examining the pre-steady-state and steady-state kinetics of the enzyme (see Glynn and Karlish, 1975b, for references). Hysteretic changes in enzyme activity have not been described, although there are suggestions that the properties of the enzyme depend not only on the composition of the assay media, but also on the handling of the enzyme prior to the assay. Kanazawa et al. (1970) employ a washing procedure prior to their transient kinetic studies, while Post et al. (1975) find that washing alters the ability of the enzyme to be phosphorylated by inorganic phosphate (P_i). Another possible example of hysteretic effects may be the slow increase in enzyme activity observed by some workers when (Na-K)-ATPase microsomes are aged (Barnett and Palazzotto, 1974; Cantley and Josephson, unpublished observations).

In this communication we have investigated hysteretic changes in ATPase activity for the (Na-K)ATPase prepared from dog kidney by the method of Jorgensen (1974). Our results indicate that the rate of ATP hydrolysis depends on a slow interconversion between forms of the enzyme with drastically different catalytic activities and that the equilibrium between forms depends on the presence of ligands required for (Na-K)ATPase activity. (Na-K)ATPase is a hysteretic enzyme as defined by Frieden (1970).

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[1] Abbreviations used: (Na-K)ATPase, Na^+ and K^+ stimulated adenosine triphosphatase; P_i, inorganic phosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PNPase, *p*-nitrophenylphosphatase; NADH, reduced nicotinamide adenine dinucleotide.