Ishikawa, H. (1978) *Nucleic Acids Res. 2*, 87–100.
Ishikawa, H., & Newburgh, R. W. (1972) *J. Mol. Biol. 64*, 135–144.
Jordan, B. R. (1975) *J. Mol. Biol. 98*, 277–280.
Jordan, B. R., Jourdan, R., & Jacq, B. (1976) *J. Mol. Biol. 101*, 85–105.
Khan, M. S. N., & Maden, B. E. H. (1977) *Nucleic Acids Res. 4*, 2495–2505.
Lava-Sanchez, P. A., & Puppo, S. (1975) *J. Mol. Biol. 95*, 9–20.
Lehrach, H., Diamond, D., Wozney, J. M., & Boedtker, H. (1977) *Biochemistry 16*, 4743–4751.
Loening, U. E. (1968) *J. Mol. Biol. 38*, 355–365.
Marrs, B., & Kaplan, S. (1970) *J. Mol. Biol. 49*, 297–317.
Miller, F. W., & Ilan, J. (1978) *J. Parasitol. 77*, 345–365.
Nazar, R. N., & Roy, K. L. (1976) *FEBS Lett. 72*, 111–116.
Nazar, R. N., Sitz, T. O., & Busch, H. (1975) *J. Biol. Chem. 250*, 8591–8597.
Payne, P. I., & Loening, U. E. (1970) *Biochim. Biophys. Acta 244*, 128–135.
Peacock, A. C., & Dingman, C. W. (1967) *Biochemistry 7*, 668–674.
Pene, J. J., Knight, E., & Darnell, J. E. (1968) *J. Mol. Biol. 33*, 609–633.
Rubin, G. M. (1973) *J. Biol. Chem. 248*, 3860–3875.
Rubinstein, L., & Clever, U. (1971) *Biochim. Biophys. Acta 246*, 517–529.
Shine, J., & Dalgarno, L. (1973) *J. Mol. Biol. 75*, 57–72.
Spohr, G., Mirault, M. E., Imaizumi, T., & Scherrer, K. (1976) *Eur. J. Biochem. 62*, 313–322.
Stevens, A. R., & Packler, P. F. (1972) *J. Mol. Biol. 66*, 225–237.
Tanaka, Y., Dyer, T. A., & Brownlee, G. G. (1980) *Nucleic Acids Res. 8*, 1259–1272.
Trapman, J., & Plants, R. J. (1976) *Biochim. Biophys. Acta 442*, 265–274.

Association of Poly(adenylate)-Deficient Messenger Ribonucleic Acid with Membranes in Mouse Kidney†

Andre J. Ouellette,* Michael B. Silverberg, and Ronald A. Malt

ABSTRACT: To describe further the metabolism of messenger ribonucleic acid (mRNA) in mouse kidney, we examined newly synthesized mRNA deficient in poly(adenylate) [poly(A)]. Approximately 50% of renal polysomal mRNA that labeled selectively in the presence of the pyrimidine analogue 5-fluoroorotic acid lacks or is deficient in poly(A) as defined by its ability to bind to poly(A) affinity columns. Nearly one-half of this poly(A)-deficient mRNA is associated uniquely with a cellular membrane fraction detected by sedimentation of renal cytoplasm in sucrose density gradients containing EDTA and nonionic detergents. Poly(A+) mRNA and poly(A)-deficient mRNA [poly(A–) mRNA] have similar modal sedimentation coefficients (20–22 S) and similar cytoplasmic distribution. Although 95% of newly synthesized poly(A+) mRNA is released in 10 mM EDTA as 20–90 S ribonucleoproteins from polysomes >80 S, only 55% of poly(A)-deficient mRNA is released under the same conditions. Poly(A)-deficient mRNA recovered from >80 S ribonucleoproteins resistant to EDTA treatment lacks ribosomal RNA, is similar in size to poly(A+) mRNA, and is associated with membranous structures, since 70% of poly(A)-deficient mRNA in EDTA-resistant ribonucleoproteins is released into the 20–80 S region by solubilizing membranes with 1% Triton X-100. These membrane-associated renal poly(A–) mRNAs could have unique coding or regulatory functions.

Although most studies of messenger ribonucleic acid (mRNA) in mammalian cells describe mRNA defined and selected on the basis of its poly(adenylate) [poly(A)][1] content (Brawerman, 1974; Molloy & Puckett, 1976), up to 60% of nonhistone mammalian mRNA may lack poly(A), is poly(A) deficient, or cannot be purified by conventional techniques involving selective affinity of poly(A) with column materials (Milcarek & Penman, 1974; Greenberg, 1976, 1977; Taylor, 1979; Van Ness et al., 1979; Ouellette, 1980). The role of mRNA that is deficient in poly(A) or lacks poly(A) [poly(A–) mRNA] is not understood in spite of its existence in many mammalian systems. For example, in cultured HeLa cells and mouse L cells, approximately 30% of total newly synthesized mRNA nominally lacks poly(A) according to its inability to bind to oligo(dT)–cellulose (Greenberg, 1976; Kaufman et al., 1977; Milcarek, 1979). mRNAs encoding myosin, protamine, and actin exist both in polyadenylated forms and in nonadenylated [or poly(A)-deficient] forms (Benoff & Nadal-Ginard, 1979; Iatrou & Dixon, 1977; Geoghegan et al., 1978). The amount and nucleotide complexity of poly(A–) mRNA in rat brain and in mouse brain are equivalent to those of poly(A+) mRNA, but its coding function is distinct (Chikaraishi, 1979; Van Ness et al., 1979).

The study of poly(A–) mRNA in mammalian organs has been limited and complicated by the high degree of toxic inhibitors required to suppress cytoplasmic ribosomal RNA (rRNA) labeling in organs of whole animals. The pyrimidine analogue 5-fluoroorotic acid (FOA) facilitates the study of poly(A–) mRNA in organs, however, because it is nontoxic

† From the Cell Biology Unit, Shriners Burns Institute, the Surgical Services, Massachusetts General Hospital, and the Department of Surgery and Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02114. *Received October 21, 1980.* Supported by National Institutes of Health Grant AM-12769 and by the Stanley Thomas Johnson Foundation. These studies were presented in preliminary form at the 11th International Congress of Biochemistry, July 8–13, 1979, Toronto, Canada.

[1] Abbreviations used: poly(A), poly(adenylate); poly(A+) mRNA, mRNA that contains poly(A); poly(A–) mRNA, mRNA lacking or deficient in poly(A); RNP, ribonucleoproteins; FOA, 5-fluoroorotic acid.

0006-2960/81/0420-3561$01.25/0

at doses that inhibit cytoplasmic accumulation of rRNA in rat liver and in mouse kidney (Wilkinson et al., 1971; Garrett et al., 1973; Ouellette & Malt, 1979).

Because the cytoplasmic accumulation of newly synthesized poly(A-) mRNA appears to be elevated during compensatory renal hypertrophy in mice (Ouellette & Malt, 1979), we used FOA to label mRNA selectively and thus to characterize poly(A-) mRNA in mouse kidney, preliminary to examining its regulation during growth. In this report, we show that over 50% of newly synthesized renal mRNA is deficient in poly(A) and that 50% of nominal poly(A-) mRNA in mouse kidney exists in rapidly sedimenting, EDTA-resistant structures and in association with membranes.

Materials and Methods

Animals and Labeling. Male mice (45 days old, 30–35 g) from Charles River Breeding Laboratories, Inc. (North Wilmington, MA) were housed under alternating 12-h cycles of light and dark and fed freely.

Radiochemicals and 5-fluoroorotic acid were administered by dorsal subcutaneous injection. To label mRNA selectively, we injected mice with 1 μmol (175.1 μg) of 5-fluoroorotic acid in 0.1 mL of H_2O 10 min before administration of radiochemicals.

Subcellular Fractionation and Preparation of RNA. Decapsulated mouse kidneys were disrupted by Dounce homogenization in ice-cold buffer consisting of 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 0.15 M NaCl, and 3 mM $MgCl_2$ (four kidneys per 3.0 mL of buffer). Nominal postmitochondrial supernatants prepared by centrifugation of homogenates at 9000 rpm for 10 min in the Sorvall SS-34 rotor were sedimented in one of three types of density gradients: (a) 36 mL of 7–47% (w/w) sucrose containing 10 mM Tris-HCl (pH 7.4), 0.50 M NaCl, and 50 mM $MgCl_2$; (b) 36 mL of 7–47% (w/w) sucrose containing 10 mM Tris-HCl (pH 7.4), 0.25 M NaCl, and 10 mM EDTA; or (c) 32 mL of 15–30% (w/w) sucrose containing 10 mM Tris-HCl (pH 7.4), 0.25 M NaCl, and 10 mM EDTA prepared over 4 mL of 2 M sucrose in the same buffer. The 7–47% (w/w) gradients were centrifuged 3 h at 26 500 rpm in the SW27 rotor at 4 °C; 15–30% (w/w) gradients were centrifuged under the same conditions except for 18 h at 22 000 rpm.

RNA was deproteinized by extraction with guanidine hydrochloride (Cox, 1969; Strohman et al., 1977; Ouellette, 1980) followed by phenol/chloroform/isoamyl alcohol (25:24:1) extraction (Perry et al., 1972). Ribonucleoprotien (RNP) fractions precipitated from gradients with 2 volumes of ethanol were dissolved in 5–10 mL of 6 M guanidine-HCl containing 25 mM EDTA (pH 7.4) and adjusted to 0.10 M potassium acetate (pH 5.0). RNA was prepared from solubilized proteins by selective precipitation with 0.5 volume of 95% ethanol. After the mixture stood at least 90 min at −20 °C, RNA was deposited by centrifugation at 9000 rpm for 15 min at 0 °C in the Sorvall SS-34 rotor, and the pellet was extracted twice more with reduced volumes of guanidine/EDTA solution. RNA from the third guanidine extraction was rinsed with 5 mL of 95% ethanol, drained briefly, dissolved in a mixture of 10 mM Tris-HCl (pH 9.0), 100 mM NaCl, 10 mM EDTA, and 0.5% sodium dodecyl sulfate, and extracted alternately with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) until the interphase was clear of denatured protein. Precipitated RNA was stored in 2 volumes of ethanol at −20 °C.

Oligo(dT)–Cellulose Chromatography. RNA extracted from renal RNP was separated into nominal poly(A)-containing and poly(A)-lacking classes by oligo(dT)–cellulose chromatography. Total RNA dissolved in 2 mL of H_2O and heated at 60 °C for 30 s was immediately placed in an ice slurry, adjusted to 10 mM Tris-HCl (pH 7.4), 0.45 M NaCl, and 0.1% (v/v) Sarkosyl NL-30 (Ciba-Geigy Corp., Greensboro, NC), and chromatographed on oligo(dT)–cellulose (Aviv & Leder, 1972; Ouellette & Malt, 1979). Poly(A+) mRNA eluted with 10 mM Tris-HCl (pH 7.4) and 0.1% Sarkosyl was similarly heated and chilled, adjusted to 0.45 M NaCl, and rechromatographed on oligo(dT)–cellulose. Under these conditions, ≤0.7% of nominal poly(A-) RNA labeled in the absence of FOA and ≤5% of nominal poly(A-) RNA labeled when FOA was present bound to oligo(dT)–cellulose when chromatographed a third time.

Sedimentation Velocity Analysis of RNA. The sedimentation properties of mRNAs were evaluated by centrifugation in linear 15–30% (w/w) sucrose density gradients containing 10 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 10 mM EDTA, and 0.5% sodium dodecyl sulfate. The positions of unlabeled nominal 18S rRNA and 28S rRNA in poly(A-) RNA detected by absorbance at 260 nm were used as reference markers. Gradients were centrifuged for 16.5 h at 23 000 rpm in the SW27 rotor at 23 °C. Radioactivity in gradient fractions was quantitated by direct counting of 1.4 mL of sample plus 2.0 mL of H_2O in 5 mL of scintillation fluid consisting of 2 volumes of xylene, 1 volume of Triton X-100 (Rohm & Haas Co., Philadelphia, PA), and 8 g of Omnifluor (New England Nuclear Corp., Boston, MA) per liter.

DEAE-Sephadex–Urea Chromatography. The inhibition of cytoplasmic rRNA labeling after administration of FOA was evaluated by anion-exchange chromatography of alkaline hydrolysates of methyl-labeled RNA. Samples of methyl-labeled poly(A-) RNA prepared by oligo(dT)–cellulose chromatography were dissolved in 3 mL of 0.3 M NaOH and were hydrolyzed for 18 h at 37 °C. Chilled samples neutralized with 3 mL of ice-cold 0.3 N perchloric acid were applied to 19 × 1.2 cm columns of DEAE-Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) equilibrated with 20 mM Tris-HCl (pH 7.4), 0.10 M NaCl, and 7 M urea (Tener, 1967; Perry & Kelley, 1974; Ouellette et al., 1975). Columns were washed with 1.5 volumes of 20 mM Tris-HCl (pH 7.4), 0.10 M NaCl, and 7 M urea; bound nucleotides were eluted with linear 200-mL gradients of 0.10–0.40 M NaCl containing 20 mM Tris-HCl (pH 7.4) and 7 M urea. Samples of 1.85 mL were assayed for radioactivity by direct counting in 1.0 mL of H_2O plus 5 mL of xylene-based scintillation fluid.

Radiochemicals and 5-Fluoroorotic Acid. [5-^{3}H]Orotic acid (11.1 Ci/mmol), L-methyl[^{3}H]methionine (5–15 Ci/mmol), and L-[^{35}S]methionine (1118.3 Ci/mmol) were purchased from New England Nuclear Corp. 5-Fluoroorotic acid (NSC-31712) was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20014.

Results

Effectiveness of 5-Fluoroorotate in Inhibiting Renal rRNA Accumulation. Accurate quantitation of newly synthesized poly(A)-deficient mRNA required an exact assessment of cytoplasmic rRNA labeling during exposure to FOA. As judged by the absence of methyl-labeled rRNA-specific dinucleotides in cytoplasmic poly(A-) RNA, accumulation of newly synthesized renal rRNA was inhibited completely in mice pretreated with FOA (Figure 1). The predominant methyl-labeled moieties in rRNA (representing approximately 85–90% of incorporated methyl radioactivity) occur at 2′-OH ribose residues (Brown & Attardi, 1965; Wagner et al., 1967) and prevent hydrolysis of the adjacent 3′–5′ phosphodiester

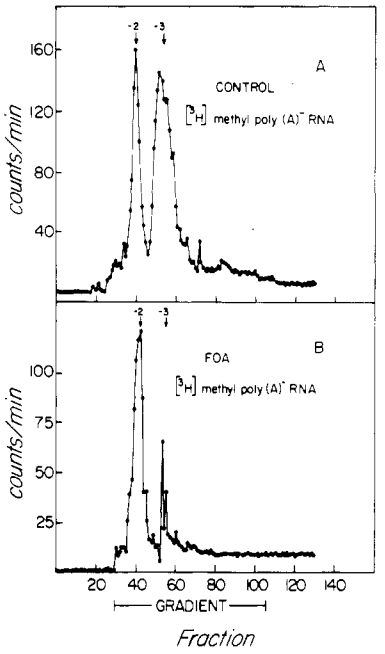

FIGURE 1: DEAE-Sephadex chromatography of methyl-labeled poly(A)-lacking RNA from mouse kidney. Mice (two per group) were injected subcutaneously with 1.25 mCi of L-[*methyl*-^{3}H]-methionine, and cytoplasmic ribonucleoproteins were deproteinized with guanidine-HCl after 90 min of labeling. High molecular weight poly(A−) RNA prepared by oligo(dT)–cellulose chromatography was hydrolyzed with NaOH, and mononucleotides (−2) and dinucleotides (−3) were separated by DEAE-Sephadex chromatography (see Materials and Methods). (A) Renal poly(A−) RNA from control mice; (B) renal poly(A−) RNA from mice labeled 10 min after injection with 1 μmol of FOA.

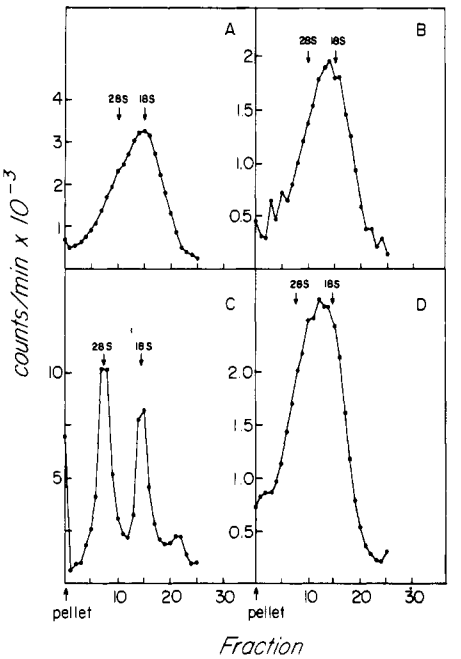

FIGURE 2: Sedimentation velocity analysis of mRNA labeled in the presence or absence of FOA. Renal postmitochondrial supernatants from mice (four per group) labeled 3 h with 250 μCi each of [^{3}H]orotic acid were sedimented in 7–47% (w/w) EDTA-containing sucrose density gradients (Figure 3A). RNA prepared from EDTA-derived 30–100S RNP from the gradients (Figure 3A, region 2) was chromatographed on oligo(dT)–cellulose, and samples of poly(A+) mRNA and poly(A−) RNA from each preparation were sedimented in sodium dodecyl sulfate containing 15–30% sucrose density gradients. Radioactivity was measured by counting total 1.4-mL gradient fractions in gel-phase xylene-based scintillation fluid. Arrows denote positions of renal nominal 28S and 18S rRNA markers. (A) Poly(A−) RNA and (B) poly(A+) mRNA from mice labeled 10 min after injection with FOA; (C) poly(A−) RNA and (D) poly(A+) mRNA from mice labeled without pretreatment with FOA.

bonds. Therefore, alkaline hydrolysis of rRNA produces 2′-O-methyl-labeled dinucleotides (net charge = −3) that can be separated by anion-exchange chromatography from mononucleotides (charge = −2) containing methyl-labeled purine rings (Tener, 1967). Since mRNA lacks methylated dinucleotides except in "caps" (Shatkin, 1976), quantitation of methyl label in the dinucleotide fraction of poly(A−) RNA provides a reliable index of continued rRNA labeling or of rRNA contamination (Perry & Kelley, 1974; Ouellette et al., 1975).

Methyl labeling of alkaline-resistant dinucleotides in renal rRNA of control mice was adequate to permit assessment of rRNA labeling in kidneys of mice treated with FOA. [^{3}H]-Methyl-labeled dinucleotides (−3 charge) predominate in poly(A−) RNA when mice are labeled without FOA (Figure 1A). A total of 67% of [^{3}H]methyl radioactivity incorporated into renal poly(A−) RNA from mice labeled with [*methyl*-^{3}H]methionine but without FOA was in dinucleotides (Figure 1A). This value was lower than the 85% figure we observed previously for mouse kidney rRNA (Ouellette et al., 1975), perhaps because hydrolysis of [*methyl*-^{3}H]methionine during storage increased incorporation of 1-carbon [^{3}H]methyl label into purine rings by de novo synthesis (Maden & Salim, 1974). Excessive nonmethyl purine ring labeling would artifactually increase the fraction of radioactivity in mononucleotides.

Continued rRNA labeling was inhibited in mice pretreated with FOA. [^{3}H]Methyl radioactivity in poly(A−) mRNA from mice labeled during exposure to FOA was only in mononucleotides (Figure 1B). The specificity of the methyl labeling pattern in newly synthesized RNA and the sedimentation patterns of selectively labeled poly(A+) mRNA and poly(A−) mRNA (Figure 2) demonstrated that FOA is completely effective in suppressing labeling of cytoplasmic rRNA, thus permitting further analysis of poly(A)-deficient mRNA in mouse kidney. Although two data points (fractions 53 and 55) in the dinucleotide region (−3 charge) apparently contained radioactivity above background (Figure 1B), these data probably do not constitute an authentic peak, since adjacent fractions contain no net radioactivity and because genuine dinucleotide peaks are symmetric and usually include at least 15 fractions (see Figure 1A). FOA administration had no effect on the mass ratio of nominal 28S rRNA and 18S rRNA.

Estimate of Newly Synthesized Poly(A)-Deficient mRNA in Kidney. Less than 50% of polysomal mRNA labeled in the presence of FOA contains poly(A) or sufficient poly(A) to permit binding to oligo(dT)–cellulose (Table I). This value was consistently observed for selectively labeled mRNA isolated from (a) total cytoplasmic RNP precipitated with $MgCl_2$ (Warner, 1966; Palmiter, 1974), (b) >80S polysomes, and (c) 20–80S mRNP derived from >80S polysomes by treatment with EDTA, the latter demonstrating association of most labeled mRNA with the translational apparatus. The fraction of selectively labeled mRNA in the poly(A)-deficient population also was similar when evaluated by poly(U)–Sepharose chromatography (data not shown; A. J. Ouellette and C. P. Ordahl, unpublished experiments). Possible selective loss of poly(A+) mRNA during extraction of RNA with phenol and chloroform also seems an unlikely explanation for the high percentage of poly(A)-deficient mRNA, since poly(A+) mRNA/poly(A−) mRNA distributions were identical when RNA was extracted solely with guanidine-HCl. In kidney, poly(A−) mRNA represents a greater fraction of newly synthesized mRNA than in HeLa cells or in mouse L cells (Milcarek et al., 1974; Greenberg, 1976). The dissimilar

Table I: Renal Content of Newly Synthesized Poly(A)-Lacking mRNA[a]

expt	conditions of labeling	radioactivity (cpm) poly(A−) RNA	poly(A+) RNA	poly(A+) (%)
1	control	133 100	47 500	26.3
	FOA	42 500	26 700	38.5
2	control	44 600	14 700	24.8
	FOA	10 500	8 600	45.1
3	control	18 400	11 500	38.4
	FOA	7 800	11 200	58.8

[a] Cytoplasmic RNAs from kidneys of control mice and of mice labeled after injection with 1 μmol of FOA were chromatographed on oligo(dT)-cellulose (see Materials and Methods). Radioactivity values denote total labeled RNA in each sample. Experiment 1: RNA from unfractionated postmitochondrial supernatants from mice labeled 2 h with 100 μCi of [^{3}H]orotic acid. Experiment 2: Mice (two per group) were labeled 3 h with 100 μCi of [^{3}H]orotic acid, and RNA was isolated from 20–80S RNP derived from >80S polysomes treated with 10 mM EDTA (see region 2 of Figure 3A). Experiment 3: RNA from unfractionated postmitochondrial supernatants from mice (two per group) labeled 3 h with 500 μCi of [^{32}P]orthophosphate.

poly(A−) mRNA content of mouse kidney and these cell lines may be attributable to the different physiologic states of the nondividing cells that chiefly comprise the renal proximal tubules with undifferentiated cultured cells growing exponentially.

Although selective inhibition of the synthesis or accumulation of poly(A+) mRNA could have reduced [^{3}H]orotic acid incorporation into poly(A+) mRNA relative to poly(A−) mRNA, incorporation of [^{32}P]orthophosphate into poly(A+) mRNA continued at the control rate in mice injected with FOA (Table I, experiment 3). Because FOA inhibits cytoplasmic rRNA accumulation by direct replacement of pyrimidines in RNA and production of unnatural polynucleotides that cannot be processed accurately (Wilkinson et al., 1971), one effect of the analogue could be to depress [^{3}H]orotate incorporation into RNA without affecting rates of synthesis. Therefore, we measured the effects of FOA on RNA synthesis directly by labeling with $^{32}P_i$ instead of [^{3}H]orotate, avoiding complications resulting from substitution of labeled pyrimidine with the analogue. As expected, the specific activity (counts per minute per microgram of RNA) of ^{32}P-labeled poly(A−) RNA was reduced nearly 4-fold in FOA-treated mice relative to controls, but this reduction reflected inhibition of cytoplasmic rRNA labeling. The specific activities of renal poly(A+) mRNA in pretreated and control mice were the same (Table I), demonstrating that FOA had little effect on the cytoplasmic appearance of poly(A+) mRNA. Although inhibition of nuclear polyadenylation by FOA cannot be discounted, selective inhibition of polymerization of purine nucleoside triphosphates by pyrimidine analogues seems a remote possibility. Furthermore, the fraction of poly(A) (assayed as mRNA radioactivity resistant to RNase T1 and RNase A) in [^{32}P]poly(A+) mRNA was the same in control or FOA-treated kidney.

Sedimentation Velocity Analysis of Poly(A)-Deficient mRNA. The sizes of selectively labeled poly(A+) mRNA and poly(A−) mRNA were compared in sucrose density gradients. Renal poly(A)-deficient RNA labeled in FOA-treated mice had the same modal sedimentation coefficient (20 S) and sedimentation profile as newly synthesized poly(A+) mRNA from the same kidneys or from control kidneys (Figure 2). In the absence of FOA, major species of labeled cytoplasmic poly(A−) RNAs (Figure 2C) in mice labeled 2 h are nominal 28S and 18S rRNAs, but no labeled rRNA could be detected in renal RNA from mice pulse labeled in the presence of FOA (Figure 2A). These data, and those in Figure 1 and in Table I, confirm that rRNA accumulation is inhibited by FOA in mouse kidney without adversely affecting mRNA synthesis. Nominal poly(A−) RNA labeled with FOA present is considered authentic mRNA because its sedimentation properties are identical with those of labeled poly(A+) mRNA, because a large fraction of nominal poly(A−) mRNA is released from polysomes by dissociation with EDTA (Table III), and because poly(A−) mRNA purified by benzoylated cellulose chromatography directs cell-free protein synthesis actively (Ouellette, 1980).

Cytoplasmic Distribution of Poly(A−) mRNA. Nominal poly(A−) mRNA and poly(A+) mRNA have the same cytoplasmic distribution when polysome structure is preserved during subcellular fractionation (Table II). In mouse kidney, HeLa cells, Sarcoma 180 cells, Vero cells, and Taper hepatoma cells, 20–60% of poly(A+) mRNA is isolated from native, "free" messenger ribonucleoproteins (mRNPs) that sediment in the 20–80S postpolysome region (Singer & Penman, 1973; Ouellette et al., 1976; Geoghegan et al., 1978; Lee & Engelhardt, 1978; McMullen et al., 1979). As shown in Table II, the distribution of newly synthesized poly(A−) mRNA and poly(A+) mRNA in polysomal (>80 S) and postpolysomal (20–80 S) RNP was equivalent, in that 24.8% of selectively labeled poly(A+) mRNA and 24.1% of poly(A−) mRNA were in postpolysomal mRNP (Table II, experiment 1). Thus, incorporation of FOA into mRNA at levels inhibiting rRNA accumulation seems not to disrupt mRNA translation since (1) the fraction of renal poly(A+) mRNA in polysomes of control and of drug-treated mice is similar (Table II, experiment 2) and (2) the sedimentation pattern of renal polysomes is unaffected by FOA dosages 10-fold greater than those used to inhibit rRNA accumulation (data not shown). These data further suggest that nominal poly(A−) mRNA and poly(A+) mRNA are translated with similar efficiency; newly syn-

Table II: Cytoplasmic Distribution of Poly(A)-Lacking mRNA[a]

expt	radioactivity (cpm) poly(A−) RNA polysomes	postpolysomes	% in postpolysomes	poly(A+) RNA polysomes	postpolysomes	% in postpolysomes
1	47 600	15 100	24.1	15 000	4 970	24.8
2				20 600	5 350	20.6
3A	24 800	27 500	51.6	5 080	24 800	85.0
3B	24 100	17 200	41.6	1 560	28 200	96.4

[a] RNA extracted from renal polysomal (>80S) and postpolysomal (20–80S) regions from mice labeled with 100 μCi of [^{3}H]orotate after injection of FOA was chromatographed on oligo(dT)-cellulose. Experiment 1: Postmitochondrial supernatant fractionated in 7–47% $MgCl_2$-containing gradients (Ouellette et al., 1976). Experiment 2: As in experiment 1, except mice were labeled in the absence of FOA. Experiment 3: Postmitochondrial supernatant was fractionated in EDTA-containing gradients, (A) as in Figure 3A and (B) as in Figure 3B.

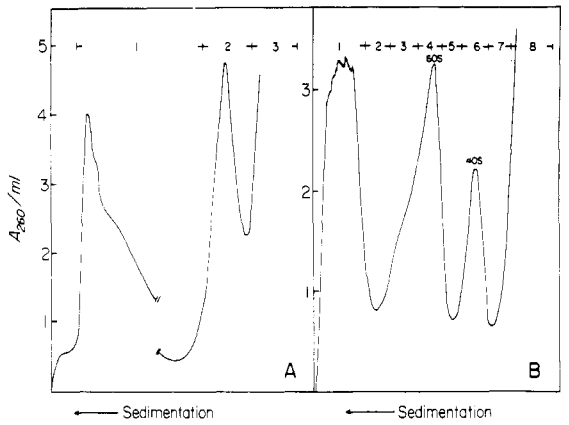

FIGURE 3: Fractionation of renal postmitochondrial supernatants in EDTA-containing sucrose density gradients. Renal postmitochondrial supernatants from unlabeled mice or from mice labeled 90 min each with 400 μCi of [^{3}H]orotic acid 10 min after FOA administration were sedimented in EDTA-containing gradients as follows: (A) in 36 mL of linear 7–47% (w/w) sucrose gradients for 3 h at 27 000 rpm in the Beckman SW27 rotor at 4 °C (see Materials and Methods); (B) in 32 mL of 15–30% (w/w) sucrose gradients prepared on 4 mL of 2 M sucrose for 18 h at 22 000 rpm in the SW27 rotor at 4 °C (see Materials and Methods). Gradients were fractionated as shown by the brackets or as described in Figure 4 and precipitated at −20 °C in 2 volumes of 95% ethanol. Interruption of the absorbance tracing in (A) indicates a doubling of the ordinate scale for the portion of the gradient to the right of the interruption.

thesized poly(A−) mRNA does not accumulate selectively in the nontranslating 20–80S mRNP fraction. The association of labeled mRNAs with polysomes in >80S structures was illustrated by the release of those mRNAs into 20–80S RNP when polysomes were disrupted with 10 mM EDTA (Table II, experiments 3 and 4), although some poly(A−) mRNA is associated with undefined structures >80S resistant to EDTA dissociation.

Sedimentation Properties of Poly(A+) and Poly(A−) Messenger Ribonucleoproteins. Poly(A+) mRNA and poly(A−) mRNA were compared with respect to the size of their corresponding mRNPs. Treatment of mouse kidney postmitochondrial supernatants with 10 mM EDTA dissociates polysomes, permitting separation of mRNPs by velocity sedimentation independent of ribosomal association (Perry & Kelley, 1966; Henshaw, 1968; Lee & Brawerman, 1971). Therefore, we measured the size of poly(A+) mRNP and of poly(A−) mRNP by quantitating radioactivity in selectively labeled poly(A+) mRNA and in poly(A)-deficient mRNA recovered from postmitochondrial RNP of mice labeled after FOA treatment and fractionated in EDTA-containing sucrose density gradients (Figure 3, Table III). As shown in Table III, 90–95% of poly(A+) mRNA was released from >80S RNP. In striking contrast to poly(A+) mRNA, however, 43% of selectively labeled poly(A)-deficient mRNA remained in rapidly sedimenting structures that were *resistant* to dissociation with EDTA (region 1, Figure 3A,B and Table III).

Sedimentation velocity analysis of RNA isolated from the *EDTA-released* mRNP fraction (Figure 3B) showed that selectively labeled poly(A+) and poly(A−) mRNAs isolated from the same gradient mRNP regions were indistinguishable in size (Figure 4, panels B, C, D, and F). Similarly, nominal poly(A−) mRNAs recovered from *EDTA-resistant* RNP were comparable in size and in heterogeneity to total poly(A+) mRNA and poly(A−) mRNA from polysomes.

Association of Poly(A)-Deficient mRNA with Rapidly Sedimenting, EDTA-Resistant Structures. Selectively labeled poly(A−) mRNA in EDTA-resistant RNP is not ribosomal in origin. As shown in Figure 4, the predominant labeled

Table III: Recovery of RNA from EDTA-Derived Ribonucleoproteins[a]

gradient region	radioactivity (cpm) poly(A−) RNA	poly(A+) RNA
1	99 700	8 800
2	33 200	12 100
3	29 200	17 700
4	20 100	17 200
5	7 700	10 600
6	12 300	12 900
7	6 250	4 230
8	1 700	1 500

[a] RNA was extracted from gradient regions shown in Figure 3B and chromatographed on oligo(dT)–cellulose. Values shown represent total RNA radioactivity in each fraction.

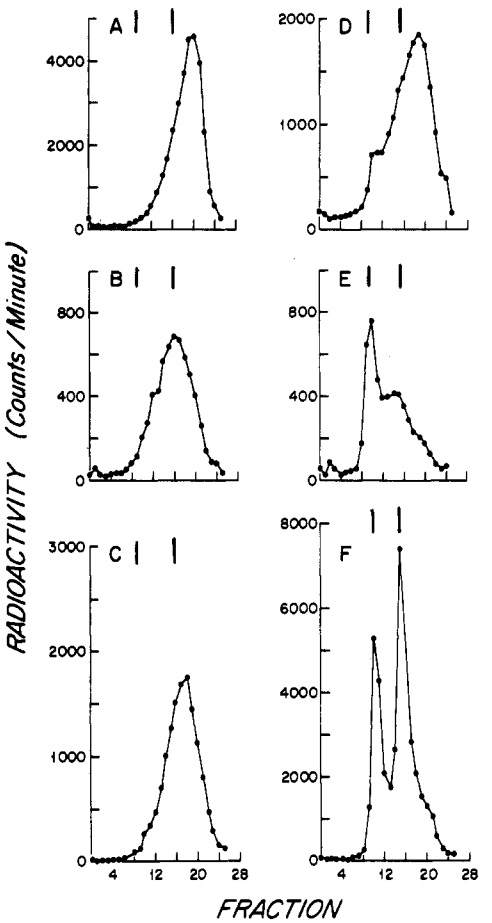

FIGURE 4: Sedimentation properties of poly(A−) RNA from >100S EDTA-resistant structures. Mice (two per group) were labeled 3 h with 250 μCi of [^{3}H]orotic acid 10 min after injection with FOA (A–C) or with 100 μCi of [^{3}H]orotic acid alone (D–F). Renal postmitochondrial supernatants were fractionated in EDTA-containing sucrose gradients as in Figure 3B, and nominal poly(A−) RNA from each region was purified by oligo(dT)–cellulose chromatography (see Materials and Methods). Poly(A−) RNA was sedimented in sodium dodecyl sulfate containing 15–30% (w/w) sucrose density gradients (Figure 2). (A and D) Poly(A−) RNA fractions corresponding to region 1 of Figure 3B; (B and E) poly(A−) RNA from gradient fractions corresponding to region 2 of Figure 3B; (C and F) poly(A−) RNA from gradient fractions corresponding to pooled regions 3–7 of Figure 3B. Vertical bars denote the positions of nominal 28S (left) and 18S (right) rRNA markers.

poly(A−) RNAs in *EDTA-released* RNP (Figure 3B, regions 2–8) in control kidney were nominal 28S and 18S rRNA (Figure 4E,F). However, pulse-labeled poly(A−) RNA from the *EDTA-resistant* fraction of control mice (region 1, Figure 3B) was mRNA-like in sedimentation pattern and showed no detectable rRNA (Figure 4D) even though rRNA in the

Table IV: Membrane Association of Renal Poly(A)-Lacking mRNA[a]

source of RNA	radioactivity (cpm) poly(A−) RNA	radioactivity (cpm) poly(A+) RNA	RNA in >80S structures (%) poly(A−) RNA	RNA in >80S structures (%) poly(A+) RNA
control gradient				
>80S RNP	124 600	11 700	52.1	12.8
20–80S RNP	114 300	79 750		
Triton X-100 gradient				
>80S RNP	23 000	6 590	19.3	5.6
20–80S RNP	96 300	111 300		

[a] One-half of the postmitochondrial supernatant from four mice labeled 3 h with 250 μCi of [^{3}H]orotic acid 10 min after injection with FOA was sedimented in an EDTA-containing 15–30% sucrose density gradient as in Figure 3B (control gradient). The remainder of the supernatant was adjusted to 1% (v/v) Triton X-100 before sedimentation in an identical gradient (Triton gradient). Gradient fractions corresponding to region 1 in Figure 3B were designated >80S RNP; gradient regions corresponding to regions 2–7 in Figure 3B were designated 20–80S. Radioactivity in RNA from each zone was quantitated by oligo(dT)-cellulose chromatography (see Materials and Methods).

EDTA-released RNP was labeled extensively (Figure 4E,F). The lack of ribosomes in >80S *EDTA-resistant* RNP and the release of nearly all poly(A+) mRNA from >80S RNP by EDTA treatment verify polysomal disaggregation by this procedure. Thus, the large fraction (~45%) of newly synthesized poly(A−) mRNA in *EDTA-resistant* RNP suggests an association of corresponding poly(A−) mRNP with additional rapidly sedimenting cytoplasmic structures.

Membrane Association of Renal Poly(A−) mRNA. Analysis of kidney cytoplasm treated with nonionic detergents demonstrated a substantial fraction of nominal poly(A−) mRNA in *EDTA-resistant* particles is in association with membranes (Table IV). Triton X-100 was added to renal postmitochondrial supernatants to solubilize membranes before sedimentation in EDTA-containing sucrose density gradients (see Figure 3B). When these samples were sedimented, the turbid, flocculent, membranous material visible in region 1 of Figure 3 and characteristic of untreated renal cytoplasm disappeared; approximately 70% of labeled poly(A−) mRNA in this region of control cytoplasm was released into the 20–80S EDTA-derived RNP fraction. However, poly(A+) mRNA and poly(A−) mRNA remain in rapidly sedimenting structures when renal postmitochondrial supernatant treated with Triton X-100 is sedimented in gradients containing $MgCl_2$ (unpublished data). Electron microscopic evidence confirmed that the microsomal membranes are totally solubilized by the detergent treatment (data not shown). The 19% of selectively labeled poly(A−) mRNA that remained resistant to EDTA may result from nonspecific aggregation of RNP particles or from an association with cytoskeletal elements (Lenk et al., 1977; Lenk & Penman, 1979).

Discussion

Over 50% of newly synthesized mRNA in mouse kidney lacks poly(A) or is poly(A) deficient, and approximately 50% of these labeled poly(A−) mRNA molecules exist in association with microsomal membranes (operationally defined). Previously, a direct association of poly(A+) mRNA with membranous elements has been demonstrated in cultured mammalian cells and in mammalian organs (Milcarek & Penman, 1974; Lande et al., 1975; Cardelli et al., 1976; Shields, 1979), but not the relation of poly(A−) mRNA to membranes. Moreover, only 5–10% of newly synthesized poly(A+) mRNA in mouse kidney exists in the rapidly sedimenting, EDTA-resistant membrane fraction. Thus, although some renal poly(A+) mRNA associates with the same EDTA-resistant structures, 90% of the mRNA that interacts with membranes in kidney nominally lacks poly(A) and represents 25% of newly synthesized mRNA in proximal tubules.

Isolation of high molecular weight poly(A−) mRNA required extraction of RNA with guanidine-HCl (see Materials and Methods). Deproteinization with phenol/chloroform/isoamyl alcohol (Perry et al., 1972; Ouellette et al., 1976) consistently resulted in extensive and *selective* degradation of nominal poly(A−) mRNA that exhibited a drastically reduced sedimentation rate (unpublished experiments).

We judge the nominal poly(A−) RNA to be mRNA by its sedimentation pattern, its continued cytoplasmic appearance in the absence of rRNA maturation, its cytoplasmic distribution, and its total nucleotide complexity. Although biologically irrelevant poly(A−) mRNA molecules could have been produced by deadenylation of membrane-associated poly(A+) mRNAs during cell disruption or during deproteinization, their generation seems unlikely since poly(A+) mRNA in EDTA-*resistant* RNP and in EDTA-*released* mRNP had similar sedimentation patterns and poly(A) contents [poly(A)/micrograms of RNA as measured by [^{3}H]-poly(U) hybridization, data not shown].

Nominal poly(A−) mRNAs in cytoplasmic EDTA-resistant RNPs probably are not nuclear RNP contaminants since the total nucleotide complexities of RNA from *EDTA-released* RNP and from *EDTA-resistant* RNP are equivalent when measured by saturation hybridization with gap-translated unique sequence mouse DNA (J. Van Ness, unpublished experiments). The nucleotide complexities of these RNAs and of unfractionated renal cytoplasmic RNA are all equal to ~2.1% of single-copy mouse DNA. Although we infer the complexity of labeled poly(A)-deficient RNA in EDTA-resistant RNP cannot exceed this 2.1% value, the possibility that this newly synthesized poly(A−) mRNA is a subset of steady-state RNA in this fraction cannot be discounted. In contrast to cytoplasmic mRNA, total renal RNA (which includes nuclear RNA) has a nucleotide complexity equal to 6.55% of single-copy DNA, the difference in complexity reflecting sequences restricted to the nucleus. If poly(A−) mRNA in *EDTA-resistant* RNP resulted from an artifact of nuclear leakage during cell disruption or during subcellular fractionation, it would exhibit a nucleotide complexity greater than that of polysomal mRNA and closer to the 6.5% nucleotide complexity value of total renal RNA.

Poly(A)-deficient mRNAs may be associated with cytoskeletal elements. Polyribosomes of HeLa cells disrupted gently with Triton X-100 remain attached to the preserved cytoskeletal framework by mRNA (Lenk et al., 1977). Although the Dounce homogenization procedure we have employed is more destructive to subcellular structures than Triton lysis, preservation of >100S renal cytoskeletal elements with attached mRNA could account for the 30% of nominal poly(A−) mRNA that persists in the >100S EDTA-resistant fractions after detergent treatment of renal postmitochondrial supernatants. The 70% of renal poly(A−) mRNA released from >100S EDTA-resistant structures by detergent treatment apparently is associated solely with membrane. Assessment of the regulatory or functional role of poly(A−) mRNA in renal cytoplasm should become more apparent as information

regarding cell-free translation products and the extent of sequence content and homology with mRNA in EDTA-released RNP becomes available.

Acknowledgments

We thank Dana Frederick for excellent technical assistance. We also thank Dr. Robert Trelstad for helpful discussions and for electron microscopy of subcellular fractions and Jeffrey Van Ness (University of Colorado Medical Center, Denver, CO) for making preliminary nucleotide complexity data available to us.

References

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

Benoff, S., & Nadal-Ginard, B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1853.

Brawerman, G. (1974) *Annu. Rev. Biochem. 43*, 621.

Brown, G. M., & Attardi, G. (1965) *Biochem. Biophys. Res. Commun. 20*, 298.

Cardelli, J., Long, B., & Pitot, H. C. (1976) *J. Cell Biol. 70*, 47.

Chikaraishi, D. M. (1979) *Biochemistry 18*, 3249.

Cox, R. A. (1969) *Methods Enzymol. 12*, 120.

Garrett, C. T., Wilkinson, D. S., & Tweedle, J. W. (1973) *Arch. Biochem. Biophys. 155*, 342.

Geoghegan, T. E., Sonenshein, G. E., & Brawerman, G. (1978) *Biochemistry 17*, 4200.

Greenberg, J. R. (1976) *Biochemistry 15*, 3516.

Greenberg, J. R. (1977) *J. Mol. Biol. 108*, 403.

Henshaw, E. C. (1968) *J. Mol. Biol. 36*, 401.

Iatrou, K., & Dixon, G. H. (1977) *Cell (Cambridge, Mass.) 10*, 433.

Kaufman, Y., Milcarek, C., & Berissi, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4801.

Lande, M. A., Adesnik, M., & Sumida, M. (1975) *J. Cell Biol. 65*, 513.

Lee, G. T.-Y., & Engelhardt, D. L. (1978) *J. Cell Biol. 79*, 85.

Lee, S. Y., & Brawerman, G. (1971) *Biochemistry 10*, 510.

Lenk, R., & Penman, S. (1979) *Cell (Cambridge, Mass.) 16*, 289.

Lenk, R., Ransom, L., & Kaufman, Y. (1977) *Cell (Cambridge, Mass.) 10*, 67.

Maden, B. E. A., & Salim, M. (1974) *J. Mol. Biol. 88*, 133.

McMullen, M. D., Shaw, P. H., & Martin, T. E. (1979) *J. Mol. Biol. 132*, 679.

Milcarek, C. (1979) *Eur. J. Biochem. 102*, 467.

Milcarek, C., & Penman, S. (1974) *J. Mol. Biol. 89*, 327.

Milcarek, C., Price, R., & Penman, S. (1974) *Cell (Cambridge, Mass.) 3*, 1.

Molloy, G., & Puckett, L. (1976) *Prog. Biophys. Mol. Biol. 31*, 1.

Ouellette, A. J. (1980) *J. Biol. Chem. 255*, 2740.

Ouellette, A. J., & Malt, R. A. (1979) *Am. J. Physiol. 237*, R360.

Ouellette, A. J., Frederick, D., & Malt, R. A. (1975) *Biochemistry 14*, 4361.

Ouellette, A. J., Kumar, A., & Malt, R. A. (1976) *Biochim. Biophys. Acta 425*, 384.

Palmiter, R. D. (1974) *Biochemistry 13*, 3606.

Perry, R. P., & Kelley, D. E. (1966) *J. Mol. Biol. 16*, 255.

Perry, R. P., & Kelley, D. E. (1974) *Cell (Cambridge, Mass.) 1*, 37.

Perry, R. P., LaTorre, J., & Kelley, D. E. (1972) *Biochim. Biophys. Acta 262*, 220.

Shatkin, A. J. (1976) *Cell (Cambridge, Mass.) 9*, 645.

Shields, D. (1979) *Biochemistry 18*, 2622.

Singer, R. H., & Penman, S. (1973) *J. Mol. Biol. 78*, 321.

Strohman, R. C., Moss, P. S., & Micou-Eastwood, J. (1977) *Cell (Cambridge, Mass.) 10*, 265.

Taylor, J. M. (1979) *Annu. Rev. Biochem. 48*, 681.

Tener, G. M. (1967) *Methods Enzymol. 12*, 398.

Van Ness, J., Maxwell, I. H., & Hahn, W. E. (1979) *Cell (Cambridge, Mass.) 18*, 1341.

Wagner, E. K., Penman, S., & Ingram, V. M. (1967) *J. Mol. Biol. 29*, 371.

Warner, J. R. (1966) *J. Mol. Biol. 19*, 383.

Wilkinson, D. S., Cihak, A., & Pitot, H. C. (1971) *J. Biol. Chem. 246*, 6418.