Processing of the Ribonucleic Acid in the Large Ribosomal Subunits of *Urechis caupo*†

Francis C. Davis* and Robin W. Mullersman

ABSTRACT: Ribosomal subunits were isolated from eggs or embryos of *Urechis caupo*, and the ribonucleic acid (RNA) was characterized by electrophoresis under denaturing conditions. The small ribosomal subunit contains a single 17S RNA sequence with a molecular weight of 6.20×10^5. The large ribosomal subunit contains four polynucleotide sequences. The 5S RNA has a molecular weight of 4.09×10^4. The 26S RNA complex isolated under nondenaturing conditions dissociates in the presence of formamide to yield a 5.8S RNA, molecular weight 5.46×10^4, and two approximately 17S and 17.5S RNA sequences with molecular weights of 6.04×10^5 and 6.61×10^5. The 17S and 17.5S RNAs of the large ribosomal subunits are formed in vivo from a 26S RNA precursor after assembly of the large ribosomal subunit. Large ribosomal subunits are transferred from the nucleus to the cytoplasm with the 26S RNA precursor intact. The hidden break to form the 17S and 17.5S RNAs is introduced in the cytoplasm. No intact 26S RNA could be detected in polysomes; this indicates that the conversion of the 26S RNA to the 17S and 17.5S RNAs may be required to produce large ribosomal subunits capable of participating in protein synthesis.

The stepwise nonconservative processing of the ribonucleic acid (RNA) transcripts in the formation of ribosomal subunits is similar for all animals (Craig, 1974; Greenberg & Penman, 1966; Rubinstein & Clever, 1971; Trapman & Planta, 1976; Griffith & Humphreys, 1979). The product in the processing is typically a small ribosomal subunit containing 18S RNA (17–18 S) and a large ribosomal subunit containing 28S (26–28 S), 5.8S, and 5S RNA species. The 18S RNA is a continuous polynucleotide (Lava-Sanchez & Puppo, 1975; Eckert et al., 1978; Miller & Ilan, 1978); however, the 28S RNA may either be a continuous polynucleotide sequence (Pene et al., 1968; Ishikawa, 1973; Lava-Sanchez & Puppo, 1975) or be two or more polynucleotides held together by hydrogen bonding (Bostock et al., 1971; Stevens & Packler, 1972; Shine & Dalgarno, 1973; Ishikawa, 1973; Lava-Sanchez & Puppo, 1975). The presence of breaks in the RNA is revealed when RNA is fractionated after denaturation (Ishikawa & Newburgh, 1972; Lava-Sanchez & Puppo, 1975; Eckert et al., 1978; Gray, 1979). The presence of hidden breaks in the RNA of the large ribosomal subunit appears to be a characteristic difference between the deuterostome and protostome phyla. All deuterostomes thus far examined have intact 28S RNA, while virtually all protostomes have 28S RNA which is fragmented by one or more hidden breaks (Ishikawa, 1977). Hidden breaks in the major RNA species of the large ribosomal subunit also have been observed in certain Protozoa (Bostock et al., 1971; Stevens & Packler, 1972; Eckert et al., 1978; Gray, 1979), higher plants (Payne & Loening, 1970; Higo et al., 1971; Grierson, 1974), and prokaryotes (Marrs & Kaplan, 1970; Doolittle, 1973). The hidden breaks present in the 28S RNA of several species have been shown to be introduced during the in vivo processing of the ribosomal RNA (Rubinstein & Clever, 1971; Ishikawa & Newburgh, 1972; Lava-Sanchez & Puppo, 1975; Jordan, 1975); however, in other species, the hidden breaks are introduced as a result of unsuppressed ribonuclease activity present during isolation (Heizmann, 1970; Ishikawa, 1975, 1975; Miller & Ilan, 1978). The sequence in the processing of RNA to introduce the break in the 28S RNA in vivo may vary from species to species. The introduction of the breaks in large ribosomal RNA of *Drosophila* has been reported to occur during nuclear processing of preribosomal particles (Jordan et al., 1976), while in *Tetrahymena* (Eckert et al., 1978) and in *Musca* (Lava-Sanchez & Puppo, 1975) the breaks occur in the cytoplasm from assembly of the large ribosomal subunits. The possible role for the introduction of the hidden breaks in the 28S RNA has not yet been established.

In the Echiuroid worm *Urechis caupo*, synthesis and processing of the ribosomal RNA (Das et al., 1970; Davis & Wilt, 1972; Das, 1976) are similar to those reported in other eukaryotes (Craig, 1974). Kinetic analysis indicates that the ribosomal genes are transcribed to yield a single 38S transcript with at least two major intermediates in the pathway to form the RNA in the ribosomal subunits, a 30S precursor to the 26S RNA of the large ribosomal subunit and a 23S precursor to the 17S RNA of the small ribosomal subunit (Davis & Wilt, 1972; Das et al., 1970; Das, 1976). As has been observed in almost all protostomes examined (Ishikawa, 1977), the 26S RNA in *Urechis* is susceptible to denaturation, yielding RNAs which sediment at about 17 and 5.8 S (Davis, 1979). In this study, the apparent molecular weights of the ribosomal RNAs are determined, and the sequence of processing of the ribosomal RNA to form functional large ribosomal subunits is characterized.

Experimental Procedures

Adult specimens of *Urechis caupo* were collected from the intertidal mud flats of Bodega Bay, CA. The procedures for maintaining the *Urechis* in the lab, collecting mature gametes, and culturing the embryos have been described previously (Davis & Wilt, 1972; Davis, 1975).

Preparation of Ribosomal Subunits. Ribosomal subunits were prepared from either eggs or gastrula-stage embryos. Eggs were collected by sedimentation at 3000*g* for 2–3 min, and subsequent steps were performed at 4 °C. Eggs were washed twice with Ca^{2+}- and Mg^{2+}-free artificial sea water (Hinegardner, 1967). NaCl was added to compensate for Ca^{2+}- and Mg^{2+}-containing salts omitted from the artificial

† From the Department of Microbiology and Cell Science, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, Florida 32611. *Received December 22, 1980.* Journal Paper No. 2787 from the Florida Agricultural Experimental Station, Gainesville, FL. Supported by U.S. Public Health Service Grant RR07021.

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sea water. Subunits were prepared after resuspending the pelleted cells in 4 volumes of 5 mM Tris-HCl (pH 7.7), 0.45 M KCl, 0.2 mM $MgCl_2$, 0.1 mM EDTA,[1] 5 mM mercaptoethanol, 0.5% Triton X-100, 0.5% sodium deoxycholate, and 1 mg/mL bentonite. Cells were lysed by five passages through a 20-gauge syringe needle followed by five passages through a 25-gauge syringe needle. The lysate was cleared by sedimentation at 12000*g* for 10 min. Approximately 1 mL of cleared lysate containing 75–80 A_{260} units was layered on 36 mL of 10–30% sucrose gradients prepared in 50 mM Tris-HCl (pH 7.7), 0.45 M KCl, 0.2 mM $MgCl_2$, and 0.1 mM EDTA. Bentonite was mixed with the sucrose at 200 μg/mL and removed from the sucrose by sedimentation at 12000*g* for 20 min prior to preparation of gradients. Gradients were centrifuged for 16 h at 27 000 rpm in a Spinco SW 27 rotor and then scanned (254 ηm) and fractionated with an ISCO gradient fractionator. Samples from which RNA was to be extracted were precipitated with 67% ethanol at −20 °C for 16–20 h.

Preparation of Polysomes. Polysomes were prepared from 24-h embryos after washing in Ca^{2+}- and Mg^{2+}-free sea water. The procedures for preparation of polysomes were the same as those used in the preparation of subunits except buffer concentrations of $MgCl_2$ and EDTA were increased to 5 and 0.5 mM, respectively. Approximately 1 mL of cleared lysate containing 75–80 A_{260} units was layered on 36 mL of 17–60% sucrose gradients in 50 mM Tris-HCl (pH 7.7), 0.45 M KCl, 5 mM $MgCl_2$, and 0.5 mM EDTA and sedimented for 3 h at 26 000 rpm in a Spinco SW 27 rotor. Gradients were fractionated and polysome fractions precipitated as described for subunits.

Preparation of Nuclei-Free Cytoplasm. Embryos were homogenized in 1.0 M glucose containing 2 mM $MgCl_2$ with a Dounce homogenizer, as previously described (Davis, 1975). The homogenate was centrifuged at 9000*g* for 3 min, and the upper two-thirds of the supernate was removed for extraction of RNA. No nuclei could be detected in the supernate, and previous studies (Davis, 1975) have shown that less than 3% of the DNA is released into the cytoplasmic fraction.

Extraction of RNA. RNA in total homogenates or cytoplasmic fractions was extracted after adding 10 volumes of 0.15 M sodium acetate (pH 5.0), 1 mg/mL bentonite, 50 μg/mL poly(vinyl sulfate) and 1% sodium dodecyl sulfate. Ethanol-precipitated subunits were pelleted at 12000*g* for 10 min, the ethanol was drained, and the pellets were resuspended in the RNA extraction buffer. Samples were extracted 3 times with phenol–chloroform–octanol (50:48:2); the aqueous phase was adjusted to 1 M NaCl and extracted once with chloroform–octanol (96:4). The initial extraction was performed at 4 °C and the remaining extractions at room temperature. RNA was precipitated in 67% ethanol at −20 °C for 16–20 h and collected by sedimentation at 12000*g* for 10 min. RNA to be analyzed by sucrose density gradient sedimentation under native conditions was dissolved at 4 °C in the gradient buffer, 0.01 M sodium acetate (pH 5.0), 0.1 M NaCl, and 0.001 M EDTA, which had been previously mixed with 0.5% diethyl pyrocarbonate and autoclaved; RNA to be analyzed by sucrose density gradient sedimentation under denaturing conditions was dissolved in 50% deionized formamide, heated to 50 °C for 3 min, and cooled to room temperature, and 9 volumes of RNA gradient buffer was added. Up to 100 μg of RNA was layered on 4.7 mL of 5–20% sucrose gradients in RNA gradient buffer. Samples were sedimented at 45 000 rpm for 2.5 h in a Spinco SW 50 rotor; gradient fractions were precipitated with 0.2 M perchloric acid, collected on glass fiber filters, and analyzed as previously described (Davis & Davis, 1978).

Polyacrylamide Gel Electrophoresis. Molecular weights of RNA species were determined by electrophoresis in polyacrylamide gels containing 98% formamide as previously described (Davis & Davis, 1978). Low molecular weight RNAs were electrophoresed at 60 °C in 10% acrylamide and 0.75% DATD at 100 V for 5 h. HeLa 5.8S RNA and *Escherichia coli* 4S and 5S RNAs were run as molecular weight markers. High molecular weight RNAs were electrophoresed at 45 °C in 2.8% acrylamide and 0.52% bis(acrylamide) at 70 V for 6 h. *Escherichia coli* 16S and 23S and HeLa 18S and 28S RNAs were run as molecular weight standards.

Labeled RNA from the large ribosomal subunit was fractionated in 2.0% acrylamide, 0.1% bis(acrylamide), and 0.5% agarose gels (0.6 × 9.0 cm) prepared in 0.04 M Tris, 0.05 M NaCl, 0.02 M sodium acetate, and 0.002 M EDTA adjusted to pH 7.8 with acetic acid (Loening, 1968). The added NaCl is required to prevent dissociation of the 26S RNA under nondenaturing conditions. Native RNA samples were dissolved in 50 μL of gel buffer supplemented with 5% glycerol and 0.008% bromophenol blue. Denatured RNA samples were dissolved in 20 μL of 50% deionized formamide, heated to 50 °C for 3 min, cooled, and diluted with a 1.67× concentrate of gel buffer–glycerol–bromophenol blue. Up to 20 μg of RNA was applied to each gel and the electrophoresis performed at 25 V for 10 h. Gels were stained with toluidine blue O and destained as described by Peacock & Dingman (1967). RNA distribution patterns were determined by scanning gels at 550 nm in a Gilford spectrophotometer equipped with a linear transport gel scanner. For determination of the distribution of labeled RNA, gels were sliced into 1.6-mm slices and placed in 7.0-mL counting vials with 0.5 mL of H_2O, the agarose was melted in 95–98 °C and incubated at 60 °C for 16–18 h, 5 mL of Instagel (Packard) was added, and fractions were counted after thorough mixing.

Results

RNA Present in Ribosomal Subunits. Previous studies of the RNA species in the ribosomes and the synthesis of ribosomal RNA in *Urechis caupo* have not characterized the size and number of species present (Davis & Wilt, 1972; Gould, 1969; Das et al., 1970). For characterization of the RNA present in the ribosomes of *Urechis*, ribosomal subunits were first isolated from homogenates of eggs by sucrose density gradient centrifugation; the RNA was extracted and analyzed by polyacrylamide gel electrophoresis either directly or after further purification of the 17S or 26S RNA by fractionation on a second sucrose gradient. The high molecular weight RNAs from the large and small ribosomal subunits were fractionated under nondenaturing and denaturing conditions (Figure 1). The small ribosomal subunit contains a single 17S species, and formamide denaturation reveals no hidden breaks. The high molecular weight RNA from the large ribosomal subunit migrates as a single 26S species under nondenaturing conditions but is denatured to yield two RNA species of unequal molecular weight, designated 17 and 17.5 S. Examination of the RNA from the small ribosomal subunit revealed no additional small molecular weight species (results not shown). In contrast, the large ribosomal subunit contains two small RNA species, 5 and 5.8 S (Figure 2). The 5.8S RNA remains hydrogen bonded in the 26S RNA complex while the 5S RNA dissociates when the ribosomal proteins are extracted.

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; DATD, *N,N'*-diallyltartardiamide; rpm, revolutions per minute; M_r, molecular weight; Cl_3CCOOH, trichloroacetic acid.

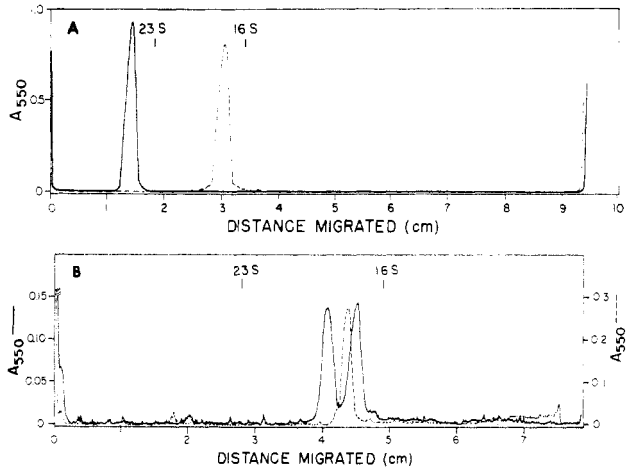

FIGURE 1: Polyacrylamide gel electrophoresis of isolated 26S and 17S ribosomal RNAs. Ribosomal subunits from eggs were isolated by sucrose-gradient sedimentation, the RNA was extracted, and the RNA that sedimented at either 26 S (—) or 17 S (---) was isolated from the second gradient. A 10-μg sample of RNA was fractionated under (A) nondenaturing conditions in 2% acrylamide, 0.1% bis(acrylamide), and 0.5% agarose gels or under (B) denaturing conditions in 4% acrylamide and 1.25% DATD gels containing 98% formamide. Gels were stained with toluidine blue O and scanned at 550 ηm. The positions of *E. coli* ribosomal RNAs on gels run in parallel are indicated.

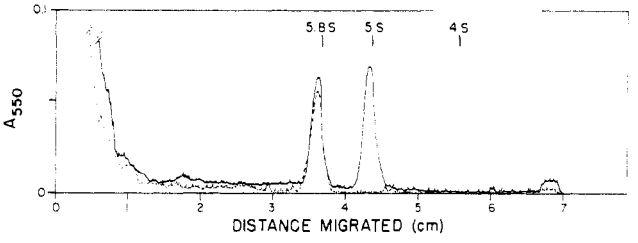

FIGURE 2: Polyacrylamide gel electrophoresis of 5.8S and 5S ribosomal RNAs. Large ribosomal subunits from eggs were isolated by sucrose-gradient sedimentation, and the RNA was extracted and fractionated on 10% acrylamide and 0.75% DATD gels containing 98% formamide. A 20-μg sample of total RNA (—) from large ribosomal subunits or 26S RNA (---) isolated by sucrose-gradient sedimentation was fractionated on each gel. The positions of *E. coli* 4S and 5S RNAs and HeLa 5.8S RNA on gels run in parallel are indicated.

The apparent molecular weight of each RNA species was determined on the basis of their electrophoretic mobility in polyacrylamide gels containing 98% formamide (Table I). For the smaller molecular weight RNA, the rate of electrophoretic migration at 60 °C was linear with the log of the molecular weight on 10% acrylamide gels for the three RNAs used as standards. The molecular weights of *E. coli* 4S RNA (Barrell & Clark, 1974) and 5S RNA (Brownlee et al., 1967) and the HeLa 5.8S RNA (Khan & Maden, 1977) were calculated from the ribonucleotide sequence. The rate of electrophoetic migration at 45 °C was linear with the log of the molecular weight for *E. coli* 16S and 23S RNAs and HeLa 18S and 28S RNAs on 2.8% acrylamide gels when the commonly cited molecular weights were used, i.e., 0.56×10^6, 1.09×10^6, 0.07×10^6, and 1.75×10^6, respectively (Loening, 1968). The molecular weights of the 17S RNA from the small ribosomal subunit and the 17S and 17.5S RNAs from the large ribosomal subunit were determined by using the molecular weights of the *E. coli* 16S and 23S RNAs calculated from the sequence analysis of the ribosomal RNA genes (Brosius et al., 1978, 1980). These values for *E. coli* ribosomal RNAs should be more accurate than the values determined by physical or electrophoretic methods.

Table I: Apparent Molecular Weights of Ribosomal RNAs[a]

RNA species	app M_r[b] ($\times 10^{-5}$)
small ribosomal subunit	
17 S	6.20 ± 0.04 (6)
large ribosomal subunit	
5 S	0.409 (8)[c]
5.8 S	0.546 ± 0.003 (8)[d]
17 S	6.04 ± 0.02 (6)
17.5 S	6.61 ± 0.06 (6)

[a] Molecular weights were determined on the basis of the relative electrophoretic mobility of RNA species under denaturing conditions compared to standards of known molecular weight. The small ribosomal RNAs were fractionated at 60 °C on 10% acrylamide and 0.75% DATD gels containing 98% formamide. The large ribosomal RNAs were fractionated at 45 °C on 2.8% acrylamide and 0.52% bis(acrylamide) gels containing 98% formamide. The rate of migration was linear with the log of the molecular weights for RNAs within the range fractionated. The molecular weight of the standards used was based either on the reported ribonucleotide sequence for *E. coli* 4S RNA, average M_r 2.5×10^4 (Barrell & Clark, 1974), *E. coli* 5S RNA, M_r 4.09×10^4 (Brownlee et al., 1967), and HeLa 5.8S RNA, M_r 5.39×10^4 (Khan & Maden, 1977), or on the ribosomal gene sequence for *E. coli* 16S RNA, M_r 5.26×10^5 (Brosius et al., 1978), and *E. coli* 23S RNA, M_r 9.91×10^5 (Brosius et al., 1980). [b] Mean ± standard deviation. Number of determinations is shown in parentheses. [c] *E. coli* and *Urechis* 5S RNAs were electrophoretically indistinguishable. [d] Molecular weight was determined with *E. coli* 4S and 5S RNAs as standards. *Urechis* and HeLa 5.8S RNAs were not electrophoretically resolved. When coelectrophoresed, broadening of the stained band indicated that the *Urechis* 5.8S RNA is slightly larger than the HeLa 5.8S RNA.

Kinetics of Labeling of RNA in Ribosomal Subunits. The two major precursors to the 26S ribosomal RNA sediment at 30 and 38 S (Davis & Wilt, 1972; Das et al., 1970). Preliminary experiments indicated that these precursors are intact polynucleotides and cannot be reduced to smaller fragments by formamide denaturation. The kinetics of labeling of the RNA in the large ribosomal subunit were examined to determine whether the breaks in the 26S RNA were introduced during in vitro processing of the ribosomal RNA or introduced in vivo either before or after the ribosomal subunits were assembled. Embryos (24 h) were labeled for 1–5 h with [^{3}H]uridine, the large ribosomal subunits were isolated from sucrose gradients, the RNA was extracted, and the 26S RNA was isolated from sucrose gradients. The purified 26S RNA was analyzed by sucrose density gradient sedimentation before and after formamide denaturation (Figure 3). After a 1-h labeling interval, most of the 26S RNA is resistant to formamide denaturation. As the labeling interval increases, a larger fraction of the 26S RNA can be denatured until at the 5-h labeling interval the majority of the newly synthesized 26S RNA can be denatured to produce the two approximately 17S RNAs. These results indicate that the RNA appears first in the large subunit as an intact 26S polynucleotide and that after assembly the break to form the two approximately 17S fragments is introduced. In addition to the labeled 26S RNA, RNA sedimenting in the 23S region of the gradient is clearly apparent at short labeling intervals under both native and denaturing conditions (Figure 3A,B). The 23S RNA is not derived from the large ribosomal subunit but is coisolated with the large ribosomal subunit due to overlapping sedimentation of the large ribosomal subunit and 23S RNA-containing ribonucleoprotein particle (see below). Analysis of the amount of label in the 26S RNA before denaturation and in the 17S and 26S RNAs after denaturation (Figure 3) is shown in Figure 4. The rate of incorporation of [^{3}H]uridine into total 26S RNA is linear from 1 to 5 h. After 5 h of continuous labeling, the incorporation into the intact 26S RNA ap-

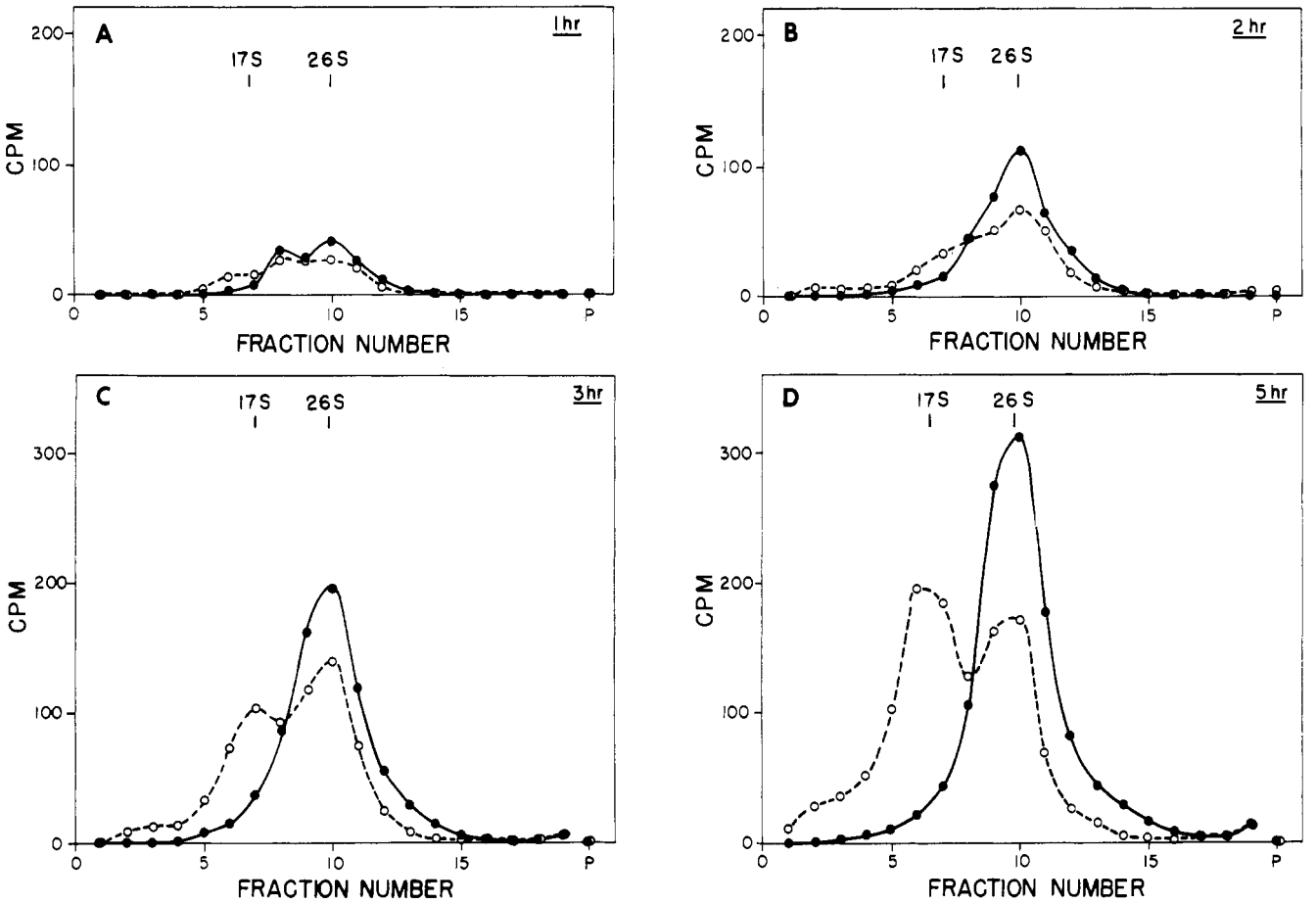

FIGURE 3: Sucrose-gradient profile of newly synthesized 26S RNA from large ribosomal subunits in 24-h embryos. Embryos were labeled with 20 μCi/mL [^{3}H]uridine. After 1 (A), 2 (B), 3 (C), and 5 h (D), embryos were collected, the large ribosomal subunits were isolated by sucrose-gradient sedimentation, the RNA was extracted, and the 26S RNA was isolated by sucrose-gradient sedimentation. Approximately 100 μg of each RNA was fractionated on a 5–20% sucrose gradient under native conditions (●—●) or after denaturation in 50% deionized formamide at 50 °C for 3 min (○—○). Gradients were sedimented, collected, and analyzed as described under Experimental Procedures. The positions of the 26S RNA and the approximately 17S RNA produced by denaturation are indicated.

proaches a plateau as the rate of formation of the hidden break approaches the rate of synthesis of the 26S RNA.

Ribonucleoprotein Particles Containing RNA Precursors. For determination of the origin of the 23S RNA present in RNA extracted from the region of a sucrose gradient containing large ribosomal subunits, the distribution of [^{3}H]uridine incorporated into ribonucleoprotein particles and ribosomal subunits was examined. Embryos (24 h) were labeled with [^{3}H]uridine for 30 min, the embryos were homogenized, and the homogenate was cleared of cell debris and centrifuged into 10–30% sucrose gradients (Figure 5). The labeled RNA sediments with a peak in the low molecular weight region of the gradient, two distinct peaks in the ribosomal subunit region of the gradient (fractions 6–15), and a heterogeneous profile in the region sedimenting more rapidly than the large ribosomal subunits. In the region of the gradient containing the ribosomal subunits, the first labeled peak sediments coincident with the small ribosomal subunit while the second labeled peak sediments slightly slower than the large ribosomal subunit. No discrete labeled species sediments coincident with the large ribosomal subunit. For examination of the labeled RNA species present, fractions in regions 1–4 in Figure 5 were pooled from duplicate gradients, and the RNA was extracted and fractionated by sucrose-gradient sedimentation. The distribution pattern of the extracted RNA from each region of the ribosomal subunit gradient is shown in Figure 6. In a 30-min labeling interval, no discrete peak of labeled 26S RNA is detectable in the fractions from the ribosomal subunit gradient. The major labeled RNA species from the large subunit region of the gradient sediments at 23 S. The label distributions of RNA species extracted from regions 1, 2, and 3 of Figure 5 are consistent with the presence of newly synthesized 17S RNA in the small ribosomal subunit and a 23S RNA in the ribonucleoprotein particle sedimenting slightly slower than the large ribosomal subunits. These results indicate that the 23S RNA species present in the RNA extracted from the large ribosomal subunits in embryos labeled for 1 or 2 h (Figure 3A,B) is not present in the large ribosomal subunit but is derived from a ribonucleoprotein particle whose sedimentation distribution overlaps with the large ribosomal subunit. Das et al. (1970) demonstrated the presence of a 23S RNA precursor to the small ribosomal RNA in the nucleoli of *Urechis* eggs. The 23S RNA-containing ribonucleoprotein particle observed here may be a precursor to the small ribosomal subunit that was released from the nucleoli in whole cell homogenates. The remaining prominently labeled RNA species sediment at approximately 30 and 38 S (Figure 6) and are present in ribonucleoprotein particles which sediment faster than the large ribosomal subunits (Figure 5). *Urechis* contains 30S and 38S ribosomal RNA precursors (Das et al., 1970; Davis & Wilt, 1972), and the ribonucleoprotein particles containing labeled RNA with these sedimentation coefficients also may be ribosomal precursor particles.

Cytoplasmic Origin of 26S RNA Processing. Variation in the cellular site for introduction of the hidden break in the 26S RNA has been reported in other protostomes (Jordan et al., 1976; Eckert et al., 1978; Lava-Sanchez & Puppo, 1975). For determination of the cellular site of introduction of hidden

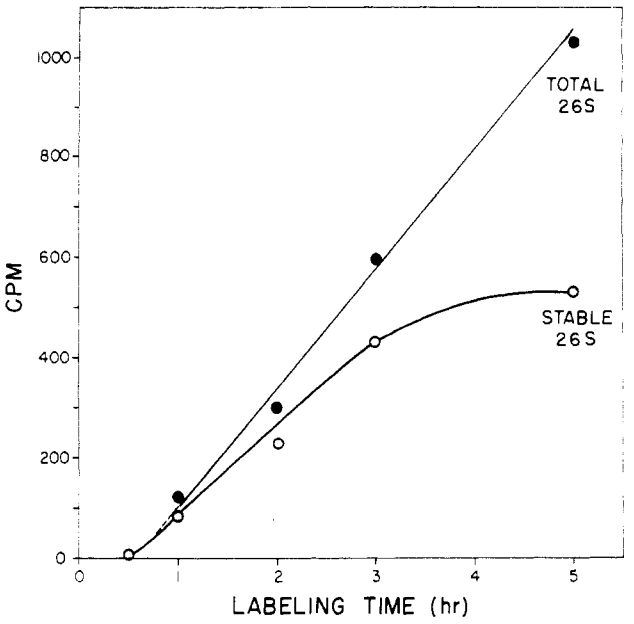

FIGURE 4: Analysis of [^{3}H]uridine incorporation into total 26S RNA and 26S RNA resistant to formamide denaturation. Total counts per minute in native 26S RNA and 26S RNA after denaturation were determined from the sedimentation profiles in Figure 3, assuming a symmetrical distribution of label in overlapping peaks. The total counts per minute were all normalized to the counts per minute in 100 μg of total 26S RNA to correct for slight variation in the amount of sample applied to each sucrose gradient.

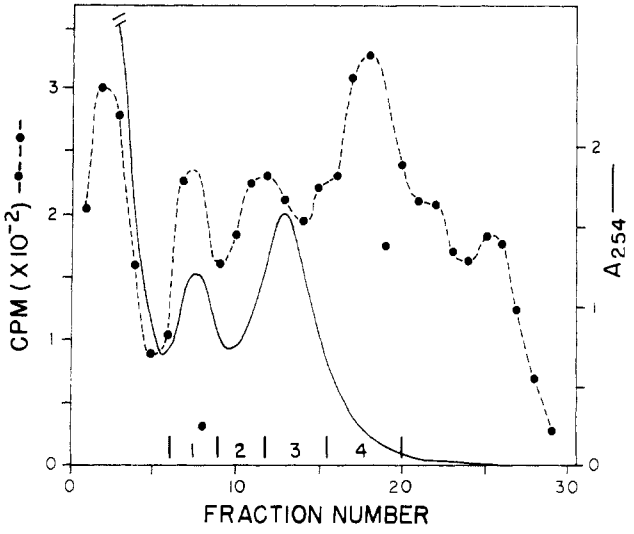

FIGURE 5: Distribution of newly synthesized RNA in ribonucleoprotein particles of 24-h embryos. Embryos were labeled with 50 μCi/mL [^{3}H]uridine for 30 min and processed as described for the preparation of ribosomal subunits under Experimental Procedures. A sample of 75 A_{260} units of the cleared homogenate was layered on a 37-mL 10–30% sucrose gradient and sedimented at 131000g for 16 h. The absorbance distribution at 254 nm (—) was monitored with an ISCO gradient fractionator and the distribution of labeled RNA (●) determined by precipitating 1.4-mL gradient fractions with 10% Cl_3CCOOH. Regions of parallel gradients which were pooled for analysis of labeled RNA content are indicated.

breaks during the processing of the 26S RNA in *Urechis*, embryos were labeled for 3 h with [^{3}H]uridine and fractionated to obtain a nuclei-free cytoplasmic fraction. Labeled 26S RNA was isolated from the cytoplasmic large ribosomal subunits and fractionated either before or after denaturation on nondenaturing polyacrylamide–agarose gels (Figure 7). Denaturation is not reversed upon exposure to nondenaturing conditions as indicated by the absence of all detectable stain in the 26S region of the gels (results not shown). The labeled cytoplasmic 26S RNA is composed of a mixture of continuous

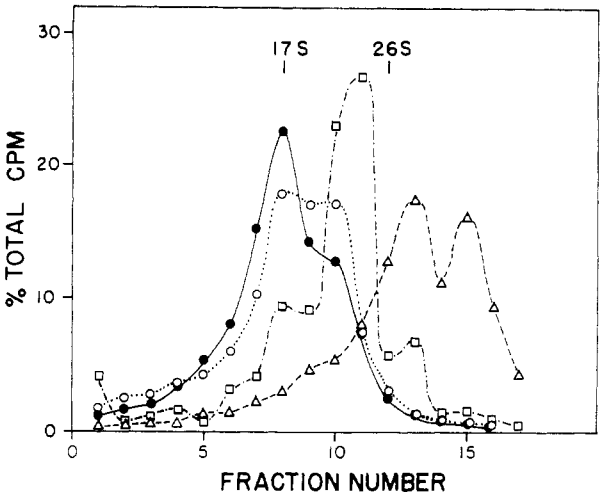

FIGURE 6: Size distribution of newly synthesized RNA extracted from regions of a ribosomal subunit gradient. RNAs from regions 1–4 of ribosomal subunit gradients (Figure 5) were pooled, the RNA was extracted and sedimented on a 4.5-mL 5–20% sucrose gradient at 243000g_{max} for 2.5 h, 0.25-mL fractions were collected, and the amount of labeled RNA in each fraction was determined after precipitation with 10% Cl_3CCOOH. The size distributions of labeled RNA from regions 1 (●—●), 2 (○···○), 3 (□···□), and 4 (△---△) are shown. The position of the 17S and 26S ribosomal RNAs was determined from the A_{254} absorbance profile. The distribution of labeled RNA is expressed as a percent of the total counts per minute in each RNA gradient.

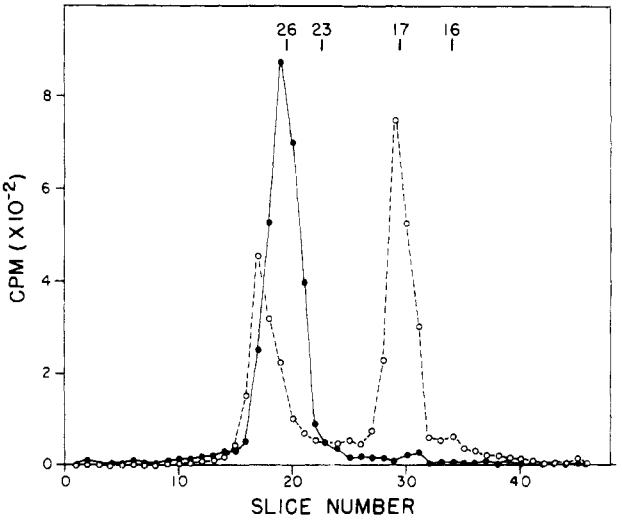

FIGURE 7: Acrylamide gel electrophoresis of newly synthesized cytoplasmic 26S RNA. Embryos (24 h) were labeled for 3 h with 50 μCi/mL [^{3}H]uridine, the large ribosomal subunits were isolated from a sucrose gradient, the RNA was extracted, and the 26S RNA fraction was isolated from a sucrose gradient. Purified 26S RNA (20 μg) was fractionated in a 2.0% acrylamide and 0.5% agarose gel. Gels were stained with toluidine blue O to locate the RNA markers and cut into 1.5-mm slices to determine the distribution of labeled RNA. The label distribution is shown for 26S RNA fractionated under nondenaturing conditions (●—●) and after denaturation at 55 °C for 1 min in 50% formamide (○---○). The RNA markers shown are the stained *E. coli* 16S and 23S ribosomal RNAs, the *Urechis* 26S RNA, and the approximately 17S RNAs produced by denaturation.

polynucleotide sequences which cannot be denatured and sequences containing a hidden break which are converted to two approximately 17S sequences after denaturation. The 17S and 17.5S RNAs are not completely resolved on this gel system, and both sequences appear as in a single peak in Figure 7. The presence of intact 26S RNA sequences in the cytoplasm indicates that large ribosomal subunits are transferred to the cytoplasm with the 26S RNA intact, and the hidden break

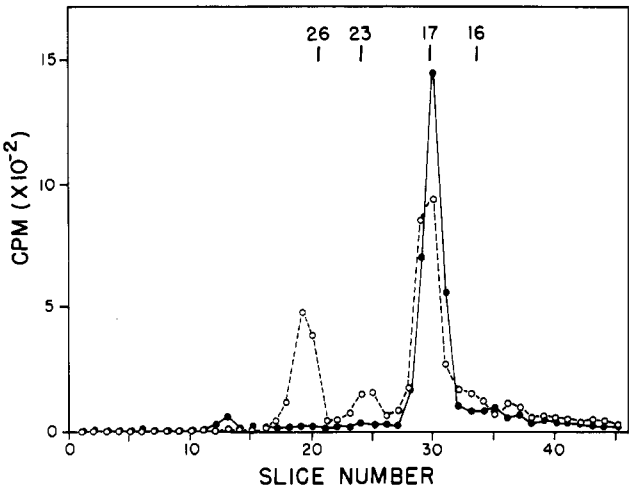

FIGURE 8: Acrylamide gel electrophoresis of newly synthesized formamide-denatured 26S RNA from native large ribosomal subunits (O---O) and polysome-derived large ribosomal subunits (●—●). Embryos (24 h) were labeled for 4 h with 50 μCi/mL [^{3}H]uridine, and the ribosomal subunits and polysomes were fractionated on a 17–60% sucrose gradient. The regions of the gradient corresponding to the native large ribosomal subunits or the polysomes (tetramer and larger) were pooled and precipitated with 67% ethanol. The large ribosomal subunits derived from polysomes were isolated from a 10–30% sucrose gradient and precipitated with 67% ethanol. RNA was extracted from the large ribosomal subunit fractions, and the 26S RNA was isolated from a 5–20% sucrose gradient. Purified 26S RNA (20 μg) was denatured and fractionated on 2.0% acrylamide and 0.5% agarose gels, and the label distribution was analyzed as described in Figure 7.

is introduced in the cytoplasm. The absence of labeled 23S RNA in the elctrophoretic pattern suggests that the procedure for preparation of the cytoplasmic fraction successfully eliminates nuclear contamination since the 23S RNA appears to be restricted to the nucleus (Das et al., 1970).

Appearance of Newly Synthesized Ribosomal Subunits in Polyribosomes. Newly synthesized large ribosomal subunits appear in the cytoplasm prior to introduction of the break that produces the two 17S RNA fragments from the 26S RNA. For determination of whether large ribosomal subunits are present in polysomes before or only after the hidden break is introduced into the 26S RNA, 24-h embryos were labeled for 4 h with [^{3}H]uridine, and the native and polysome-derived large ribosomal subunits were isolated from homogenates of whole embryos. Analyses of denatured 26S RNA present in large ribosomal subunits derived from polysomes and native large ribosomal subunits are shown in Figure 8. All of the label in the 26S RNA from derived subunits is converted to the two 17S fragments, while a portion of the labeled 26S RNA from native subunits remains intact after denaturation. These results indicate that the intact 26S RNA appears in native large ribosomal subunits and the nick to produce the two 17S fragments introduced prior to the appearance of the large subunits into polysomes.

Discussion

Examination of the RNA present in *Urechis* ribosomal subunits revealed that several RNA species are present. The small ribosomal subunit contains a single RNA species (Figure 1B) which is typical of all eukaryotes (Ishikawa, 1977). The molecular weight of the RNA from the small ribosomal subunit is 6.20×10^5 (Table I). The RNA of the large ribosomal subunit is composed of four RNA species (Figures 1B and 2; Table I). The 5S RNA, molecular weight 4.09×10^4, readily dissociates from nondenaturing conditions during extraction of the ribosomal proteins (Figure 2). The molecular weight of the 5S RNA is consistent with an RNA sequence containing 120 nucleotides, similar to the 120 or 121 nucleotides that have been observed in other eukaryotes (Barrell & Clark, 1974). The 26S RNA species isolated under nondenaturing conditions is composed of three RNA species which dissociate after formamide denaturation (Figures 1 and 2). The 5.8S RNA, molecular weight 5.46×10^4, is similar in size to corresponding RNAs from other eukaryotes which have been reported to be composed of 158–163 nucleotides (Khan & Maden, 1977; Rubin, 1973; Nazar et al., 1975; Nazar & Roy, 1976; Tanaka et al., 1980). The two larger RNA species have molecular weights of 6.04×10^5 and 6.61×10^5. The presence of one or more hidden breaks in the major RNA species of the large ribosomal subunit is characteristic of members of the animal phyla classified as protostomes. As in *Urechis*, a break at or near the center of the major RNA species has been observed in a number of these organisms (Ishikawa, 1973, 1977; Shine & Dalgarno, 1973) while in other protostomes more than one hidden break may be present (Lava-Sanchez & Puppo, 1975).

Molecular weights of *Urechis* ribosomal RNAs were determined by measurement of the relative rate of electrophoretic migration under denaturing conditions compared to standards of known molecular weight (Table I). Complete denaturation of RNA may not be achieved in the presence of formamide at room temperature; however, a linear rate of migration vs. the log of the molecular weight has been achieved at elevated temperatures for RNA species resistant to denaturation at room temperature (Lehrach et al., 1977; Spohr et al., 1976). In preliminary experiments, the relative rate of migration of the standard RNAs and the *Urechis* ribosomal RNAs was examined at room temperature and at elevated temperatures to 60 °C. Changes in the relative rates of migration of RNAs were observed at elevated temperatures. The small RNA species were fractionated at 60 °C although denaturation appeared to be complete at lower temperatures. For the larger ribosomal RNA species, an elevated temperature of 45 °C or higher appeared to be required to achieve a constant relative rate of migration for the standard RNA species and the *Urechis* ribosomal RNAs. Electrophoresis at 45 °C was used for the molecular weight determinations since denaturation appeared complete at this temperature, and at higher temperatures the RNA bands in the polyacrylamide gels became increasingly distorted, and degradation of the larger RNA species was apparent.

The molecular weight of the standards used was based either on the molecular weight determined by RNA sequence analysis of the *E. coli* 4S and 5S RNAs and HeLa 5.8S RNA (Barrell & Clark, 1974; Brownlee et al., 1967; Khan & Maden, 1977) or on the ribosomal gene sequence analysis for the *E. coli* 16S and 23S RNAs (Brosium et al., 1978, 1980). The molecular weights calculated for the *E. coli* 16S and 23S RNAs are lower than the molecular weights commonly cited for these RNA species (Loening, 1968). Consequently, the values calculated for the molecular weight of the larger ribosomal RNA species in *Urechis* appear lower than values reported for other invertebrates calculated from their electrophoretic mobilities relative to the same standards but using the higher molecular weights for the standards. For comparison, the molecular weights of the *Urechis* large ribosomal RNAs were determined under nondenaturing conditions and by use of molecular weights for *E. coli* 16S and 23S RNAs and HeLa 18S and 28S RNAs of 0.56×10^6, 1.07×10^6, 0.70×10^6, and 1.75×10^6, respectively (Loening, 1968). Under nondenaturing conditions, the apparent molecular weight of the 17S RNA from the small ribosomal subunit is 0.67 (±0.01 SD) $\times 10^6$

(unpublished experiments). The intact 26S RNA has an apparent molecular weight of 1.36 (±0.02 SD) × 10^6 (unpublished experiments). The values for 17S RNA from the small ribosomal subunit and the 26S RNA fall within the range of values reported for other lower eukaryotes which were determined with similar methods (Loening, 1968; Ishikawa, 1977).

In *Urechis* embryos, newly synthesized RNA appears in the small ribosomal subunits more rapidly than it does in the RNA of the large ribosomal subunits. Less than 30 min is required for newly synthesized 17S RNA to appear in small ribosomal subunits while between 30 min and 1 h is required for synthesis and processing before the newly synthesized 26S RNA appears in large ribosomal subunits (Figures 3 and 6). The slower processing of the RNA in the large ribosomal subunit appears to be a phenomenon common to most eukaryotes (Craig, 1974). The difference in the rate of processing of newly synthesized RNA to form the 17S and 26S RNAs was previously observed in *Urechis* unfertilized eggs (Das, 1976); however, the rate of processing of the RNA in both ribosomal subunits is much slower in the eggs than in the embryos (Gould, 1969; Das et al., 1970; Das, 1976).

The newly synthesized 26S RNA appears in the large ribosomal subunits first as a continuous polynucleotide sequence resistant to denaturation (Figure 3). During 5 h of continuous labeling, the amount of label in the intact 26S RNA approaches a plateau as the rate of conversion of the 26S into the 17S and 17.5S RNAs approaches the rate of incorporation of label into newly synthesized 26S RNA (Figure 4). Since the rate of incorporation of [^{3}H]uridine into the total RNA of the large ribosomal subunits is essentially linear over the interval examined, the rate of conversion of label from the intact 26S RNA to the 17S and 17.5S RNAs should correspond to the actual rate of conversion. The $t_{1/2}$ of large ribosomal subunits containing intact 26S RNA can be calculated if it is assumed that during the interval examined (1) the specific activity of the precursor pool is constant, (2) the rate of synthesis of 26S RNA is constant, and (3) the processing of the 26S RNA to form the 17S and 17.5S RNAs is a random event. Using the slope of the curve for incorporation of label into the RNA of the large ribosomal subunit as the rate of synthesis and the total counts per minute in the 26S RNA at 5 h as the steady-state level of label in the intact 26S RNA (Figure 4), we calculated the $t_{1/2}$ of the pool of large ribosomal subunits containing intact 26S RNA as approximately 1.6 h.

The measurement of the appearance of label first in the 26S RNA followed by its conversion to the 17S and 17.5S RNAs demonstrated that the introduction of the hidden break is a step in the in vivo processing of the RNA. The introduction of the hidden break after formation of the 26S RNA is at the same stage in the processing of the ribosomal RNA as has been observed for three other organisms, *Drosophila* (Jordan et al., 1976), *Musca* (Lava-Sanchez & Puppo, 1975), and *Tetrahymena* (Eckert et al., 1978). In *Urechis*, the hidden break in the 26S RNA is introduced after the large ribosomal subunits appear in the cytoplasm, as occurs in *Musca* (Lava-Sanchez & Puppo, 1975) and *Tetrahymena* (Eckert et al., 1978), rather than in the nucleus before the precursor particles are transferred to the cytoplasm, as occurs in *Drosophila* (Jordan et al., 1976).

Previous studies have not established a role for the introduction of the hidden breaks in the 26S RNA. It has been speculated that the breaks may either be formed as a random event in the aging of the ribosomal subunits and have nothing to do with their function in protein synthesis or be formed as a required maturation event to produce the functionally active subunits (Lava-Sanchez & Puppo, 1975; Jordan et al., 1976; Eckert et al., 1978; Miller & Ilan, 1978). The presence of a mixed population of large ribosomal subunits in the cytoplasm, some with the 26S RNA intact and others with 17S and 17.5S RNA sequences, provided an opportunity to determine whether the introduction of the hidden break played a role in the formation of functional large ribosomal subunits. Examination of the denatured RNA from native large ribosomal subunits and from polysome-derived large ribosomal subunits revealed that the newly synthesized RNA from the native subunits was a mixture of 26S RNA and 17S and 17.5S RNAs while the polysome-derived subunits contained only 17S and 17.5S RNAs (Figure 8). The absence of intact 26S RNA in the polysome-derived subunits is interpreted to indicate that the introduction of the hidden nick in the 26S ribosomal RNA is a requirement for formation of functional large ribosomal subunits.

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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.

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