Double-Stranded Ribonucleic Acid in Sea Urchin Embryos†

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ABSTRACT: Double-stranded RNA (ds-RNA) has been purified from hatching blastulae of the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus* using selective nuclease digestion and chromatography on cellulose columns. The ds-RNA is synthesized in nuclei at approximately 0.2% of the rate of synthesis of nuclear RNA; it appears to be confined to the nucleus and behaves kinetically as rapidly turning-over nuclear RNA. The ds-RNA is not an artifact of extraction with sodium dodecyl sulfate and phenol, does not consist of degradation products of rRNA and is distinguishable from DNA–RNA hybrids. Some of the ds-RNA can be isolated from large, ribonuclease-sensitive RNA molecules. Ribonuclease-resistant [^{32}P]RNA does not display a "symmetric" base composition but contains duplexed RNA sequences of symmetric base content associated with sequences extremely rich in adenylate.

Current interest in viral RNA metabolism and stimulation of production of interferons by animal cells has led to recognition of double-stranded ribonucleic acid (ds-RNA) in presumably uninfected cells (Colby and Duesberg, 1969; Stern and Friedman, 1970, 1971). Using microcomplement fixation by specific anti-ds-RNA sera small amounts of antigen can be detected in uninfected BHK and KB cells (Stollar and Stollar, 1970). Both observations are in accord with the report by Montagnier (1968) that ribonuclease-resistant RNA with some of the properties of a duplex molecule can be isolated from ascites cells, chick embryo fibroblasts, BHK and rat liver cells, in addition to BHK cells and chick fibroblasts transformed by the Rous sarcoma virus. ds-RNA thus appears ubiquitous in the cells of warm-blooded animals and may in fact be present in all eukaryotic cells, perhaps as a consequence of inapparent viral infection; alternatively, ds-RNA may reflect some heretofore unnoticed function in cellular RNA metabolism. There is insufficient information regarding the metabolic properties of these molecules to establish the role of ds-RNA in viral or cellular functions.

In order to extend the class of cells known to synthesize ds-RNA we have utilized early embryos of the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus*. The occurrence of duplex polyribonucleotides has not been reported in invertebrate embryos of any species. In addition, quantitative data on the absolute rates of synthesis and degradation of the various classes of cellular RNA have been obtained using sea urchin embryos (Emerson and Humphreys, 1970, 1971; Brandhorst and Humphreys, 1971, 1972) making it possible to relate the metabolic properties of cellular ds-RNA to those of a previously recognized class of cellular RNA molecules.

Experimental Section

Embryos. Sea urchin embryos (*S. purpuratus* and *L. pictus*) were grown at 18° with at least 95% normal development according to Emerson and Humphreys (1970) until 50% had hatched (15 ± 0.5 hr).

Labeling. Embryos were collected by centrifugation for 15 sec at 100*g* and resuspended in fresh antibiotic sea water (Emerson and Humphreys, 1970) to 1–10% (v/v) and radioactive materials were added as specified in individual experiments.

Isolation and Fractionation of RNA. Embryos were collected and washed twice by low-speed centrifugation through acid sea water (Emerson and Humphreys, 1970) at 0°, except for experiments in which nuclei and cytoplasm were to be separated (see below). Labeled embryos (per 0.4–0.7 ml of packed volume) were resuspended in 10–15 volumes of lysis buffer (0.20 M NaCl, 10 mM Na_2EDTA, 10 mM Tris-HCl, 10 mM β-mercaptoethanol, and 0.5% w/v sodium dodecyl sulfate, pH 7.40) at 37° and predigested Pronase stock solution (10 mg/ml, Calbiochem) was added to give 0.5 mg/ml of Pronase; the embryos were homogenized with 15 strokes in a Dounce homogenizer with a tight-fitting pestle and incubated 30 min at 37°. The homogenate was extracted three times for 10 min at 37° with equal volumes of distilled phenol which had been saturated with standard buffer (0.20 M NaCl, 1 mM Na_2EDTA, and 10 mM Tris-HCl, pH 7.40). Phenol phases were pooled and extracted with 0.5 volume of standard buffer. The pooled aqueous phase was brought to 0.1 M in sodium acetate and 65% in ethanol and stored overnight at −20°.

The precipitated nucleic acid was collected by centrifugation for 15 min at 27,000*g* at 4°, rinsed with 95% ethanol at −20°, and dried in a stream of air. The pellet was dissolved in sufficient DNase buffer (50 mM NaCl, 2 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.40) to yield an A_{260} of 8–16 cm^{-1}. Ribonuclease-free pancreatic deoxyribonuclease stock (1 mg/ml) was added to give 20 μg/ml of DNase and the solution was incubated for 30 min at room temperature. The solution was brought to 0.25 M *total* NaCl by adding 5.0 M NaCl, then 20 μg/ml of pancreatic RNase (Sigma) and 20 unit/ml of T1 RNase (Worthington) were added and the solution was incubated 30 min at 37°. The reaction was terminated by thrice extracting 10 min at 37° with an equal volume of buffer-saturated phenol. The phenol phase was reextracted and the nucleic acid precipitated as above.

The precipitated nucleic acid was dissolved in 0.5 ml of TSE buffer (0.10 M NaCl, 1 mM Na_2EDTA, and 50 mM Tris-HCl, pH 6.90) to give an A_{260} not exceeding 20 cm^{-1} and ethanol was added to 35% (v/v). The sample was applied to a cellulose column (Franklin, 1966) and the column was

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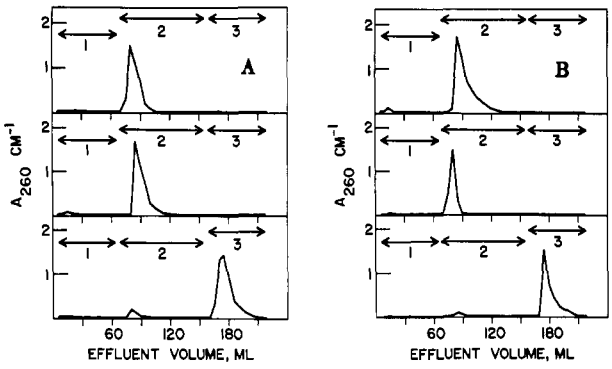

FIGURE 1: Cellulose column optical density elution profiles. (A) For polyriboadenylate (top), polyribouridylate (middle), and poly(rA):poly(rU) duplex (bottom). Duplexes were prepared and homopolymers chromatographed as described in the Experimental Section. (B) For polyriboinosinate (top), polyribocytidylate (middle) and poly(rI):poly(rC) duplex (bottom).

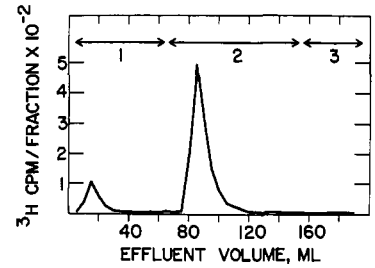

FIGURE 2: Cellulose column radioactivity elution profile for ds-[^{3}H]RNA after heating and rapid cooling.

washed with TSE–ethanol (65:35, v/v); all column buffers were previously degassed by stirring under reduced pressure. Fractions of 5-ml were collected with a volumetric device. After collecting 15 fractions the column was washed with TSE–ethanol (85:15, v/v) and finally with TSE alone. For each fraction the absorbance at 260 nm was noted and the acid-precipitable radioactivity determined. Representative fractions for each peak were pooled and characterized further.

Homopolymer (Miles) duplexes were prepared in SSC (0.15 M NaCl–0.015 M sodium citrate, pH 7.0) by annealing polyribouridylate (poly(U)) with polyriboadenylate (poly(A)) and polyribocytidylate (poly(C)) with polyriboinosinate (poly(I)). These duplexes and also the single-stranded homopolymers were tested for hypochromicity and stability toward pancreatic RNase and were chromatographed as described for embryo RNA. In each case (Figure 1A,B) duplex ribopolymers chromatographed with the third peak while single-stranded polymers chromatographed with the second peak. Similarly (Figure 2), double-stranded embryo RNA chromatographed with the second peak after denaturation at 100° and rapid cooling.

Base Composition Analysis. ^{32}P base compositions of various RNA fractions were determined after precipitation with 0.5 M $HClO_4$ (Emerson and Humphreys, 1971).

Cell Fractionation. Cells were fractionated into nuclei and cytoplasm by a procedure developed by Dr. B. P. Brandhorst (Brandhorst and Humphreys, 1972). Packed embryos (0.1 ml) were suspended in 1 ml of sea water (Emerson and Humphreys, 1970) and layered on 10 ml of 1.5 M dextrose and then centrifuged for 5 min at top speed in a clinical centrifuge. The pellet was resuspended in 4 ml of 1.5 M dextrose and centrifuged for 1 min at 250*g*. This washing was repeated until the embryos began to dissociate into cells as evidenced by turbidity in the supernatant. They were pelleted and resuspended in 3 ml of fresh TNM buffer (0.24 M NH_4Cl, 5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.8) containing 0.5% (v/v) Triton X-100 and triturated with a Pasteur pipet. The preparation was examined under phase optics to determine that all cells were lysed, layered on 2 ml of 1.0 M sucrose containing 5 mM $MgCl_2$, and centrifuged in a Sorvall swinging-bucket rotor at 4000 rpm. The pelleted material was nuclei with less than 1% of cytoplasm and the supernatant above the sucrose layer was cytoplasm with 5–10% nuclear contamination.

Polysomes in the cytoplasmic fraction were resolved on 37-ml, 15–35% (w/v) linear sucrose gradients in HSB buffer (0.40 M KCl, 10 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.6). In some cases the cytoplasmic fraction was brought to 50 mM in Na_2EDTA and fractionated on gradients containing 50 mM Na_2EDTA. Gradients were centrifuged 3 hr at 3° at 25,000 rpm in the Spinco SW-27 rotor and monitored by pumping through a continuous-flow cell in a Gilford Model 2000 spectrophotometer. Fractions were collected, mixed with 50 μg of yeast RNA, and assayed for total and alkali-stable, acid-insoluble radioactivity. Other gradients were divided into fractions containing material sedimenting faster than monoribosomes, or slower than and including monoribosomes. These were mixed with carrier RNA and precipitated with ethanol, and ds-RNA prepared as from original extracts of RNA.

Radioactivity Determinations. Extraction and purification of ds-RNA were assayed by determining total and alkali-stable, acid-insoluble radioactivity. Labeled RNA was assumed to be the difference. Hydrolysis was achieved in 0.3 M NaOH at 37° for 18 hr. Aliquots before and after hydrolysis were brought to 7.5% (w/v) trichloroacetic acid after addition of 200 μg of yeast RNA, chilled for 15 min on an ice bath, collected on 0.45 μ Millipore filters, and washed with excess cold 5% trichloroacetic acid. RNA was eluted from the filters with 0.3 ml of 0.3 M NaOH for 20 min at room temperature and counted directly in 90% (v/v) toluene, 3% Beckman Bio-Solv 2, and 7% Bio-Solv 3 containing 0.4% (w/v) 2,5-diphenyloxazole and 0.005% 1,4-bis[2-(5-phenyloxazolyl)]benzene.

Results

ds-RNA in Sea Urchin Embryos. Embryos were labeled beginning at 15-hr postfertilization for 30 or 240 min using 10 μCi/ml of [5-^{3}H]uridine (Schwarz, 28.0 Ci/mmole) and RNA was extracted, digested, and chromatographed on cellulose to purify ds-RNA. Radioactivity elution profiles for embryos labeled 30 and 240 min are shown in Figure 3A,B, respectively. The fraction of labeled RNA which was double stranded was computed assuming 100% recovery of ds-RNA during the second phenol extraction and using the data of Tables I and II. The radioactive material eluting in the third chromatographic peak appeared to be RNA since it was hydrolyzed completely in 0.3 M NaOH and since over 99% of the radioactivity became ribonuclease sensitive following heating. The latter samples were heated 3 min at 100°, chilled quickly to 0°, brought to 0.25 M total NaCl, and digested 30 min at 37° with 20 μg/ml of pancreatic ribonuclease. The ribonuclease resistance of all three peaks in Figure 3B was determined (Table II). Although the material eluting in the third peak is approximately 90% resistant to pancreatic RNase under con-

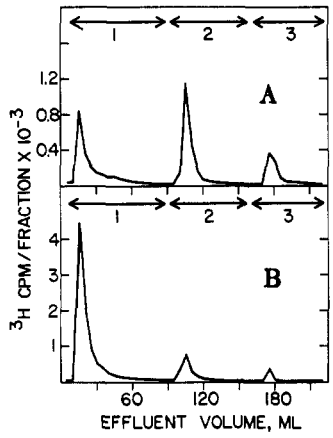

FIGURE 3: Cellulose column radioactivity elution profile for [^{3}H]-RNA after ribonuclease digestion as described in the Experimental Section. (A) Embryos labeled for 30 min; (B) embryos labeled for 240 min.

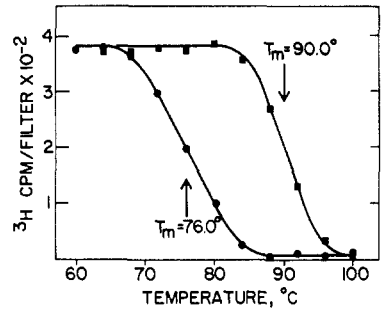

FIGURE 4: Melting curves of ds-[^{3}H]RNA determined as described in the text. (●) 0.1 × SSC, and (■) SSC.

ditions in which ss-RNA (single stranded) is completely solubilized, after denaturation it becomes completely RNase sensitive. In contrast the RNase sensitivity of material in the first two peaks is not changed by heating, indicating little or no ds-RNA.

The thermal denaturation of the RNA eluting in the third peak was measured. ds-RNA was prepared from 15-hr embryos labeled for 240 min with 15 μCi/ml of [^{3}H]uridine, precipitated in ethanol in the presence of yeast RNA, dried, and then dissolved in SSC or 0.1 × SSC. Aliquots (0.5 ml) were raised to various temperatures for 5 min, chilled rapidly on an ice–water bath, brought to 0.25 M total NaCl, and incubated 30 min at 37° with 20 μg/ml of pancreatic RNase. Carrier RNA was added and the acid-insoluble radioactivity determined (Figure 4). The denaturation, as judged by the disappearance of resistance to pancreatic ribonuclease, is sharp. The T_m is higher at the higher ionic strength as expected for a duplex polynucleotide: in SSC the T_m is 90° whereas in 0.1 × SSC the T_m is 76°. Renaturation, as reappearance of RNase resistance, was never observed, even in samples heated for 3 min at 100°, brought to 0.25 M NaCl, incubated for 60 min at 37°, and then treated with ribonuclease. Evidently this material does not consist of self-complementary single strands.

Complementary but largely ss-RNA sequences may anneal to a double-helical, RNase-resistant conformation during extraction with phenol, sodium dodecyl sulfate, and Pronase (Weissmann *et al.*, 1968). This effect was checked by treating freshly homogenized embryos directly with ribonuclease. Embryos (15 hr) were labeled for 30 or 240 min with 10 μCi/ml of [^{3}H]uridine, collected, and homogenized in standard buffer containing 50 μg/ml of pancreatic RNase, then incubated at 37°. After 40 min the homogenate was brought to 0.5% in dodecyl sulfate, extracted with phenol, and precipitated as above. The pellet was dissolved in DNase buffer and incubated for 30 min at room temperature with 20 μg/ml of DNase. The solution was extracted, precipitated, and chromatographed as above (Figure 5 and Table III). By comparison to Table I it is evident that immediate digestion with ribonuclease does not change the yield of ds-RNA. This suggests that a small proportion of the rapidly turning over RNA in sea urchin embryos occurs *in vivo* as RNA duplexes.

The occurrence of complementary but nonduplexed RNA sequences was examined by annealing labeled embryo RNA prior to nuclease digestions. Embryos were labeled at 15-hr postfertilization for 30 or 240 min with 20 μCi/ml of [^{3}H]-uridine and nucleic acid was extracted and precipitated with ethanol. The pellet was redissolved in 2 × SSC to a concentration of 300 μg/ml. Aliquots were sealed in glass ampoules and incubated 48 hr at 72°. Control and annealed preparations of RNA were then digested and chromatographed as usual for ds-RNA. The annealing procedure doubled the

TABLE I: Purification of ds-RNA.[a]

	Duration of Pulse	
	30 min	120 min
a. RNA radioactivity treated with nucleases	9.56×10^5	3.90×10^6
b. RNA radioactivity after digestion placed on column	1.91×10^4	5.82×10^4
c. RNA radioactivity in third chromatographic peak	1.69×10^3	2.60×10^3
d. RNase-resistant RNA radioactivity in third peak	1.53×10^3	2.34×10^3
As per cent of step c	90.4	89.8
e. ds-RNA radioactivity, as per cent of step a	0.16	0.06

[a] Acid-insoluble, alkali-labile radioactivity, cpm.

TABLE II: Ribonuclease-Resistance of Chromatographic Peaks (Figure 3B).[a]

	Chromatographic Fraction		
	1	2	3
a. Alkali-labile radioactivity	30,440	8564	2064
b. Acid-insoluble radioactivity after RNase	1,724	596	1828
c. Acid-insoluble radioactivity after denaturation plus RNase	1,508	88	
d. RNase resistance = $(c - d)/(b) \times 100\%$	0.7	5.9	89.9

[a] Acid-insoluble radioactivity, cpm.

TABLE III: Purification of ds-RNA after Homogenization Directly with Ribonuclease.[a]

	Duration of Pulse	
	30 min	120 min
a. RNA radioactivity before RNase	9.11×10^5	3.44×10^6
b. RNA radioactivity after RNase	2.61×10^4	1.06×10^5
As per cent of step a	2.5	2.5
c. RNA radioactivity placed on column	1.99×10^4	5.00×10^4
d. RNA radioactivity in third peak	1.74×10^3	2.24×10^3
e. RNase-resistant RNA radioactivity in third peak	1.64×10^3	2.06×10^3
As per cent of step d	93.9	92.0
f. ds-RNA radioactivity, as per cent of step a	0.18	0.06

[a] Acid-insoluble, alkali-labile radioactivity, cpm.

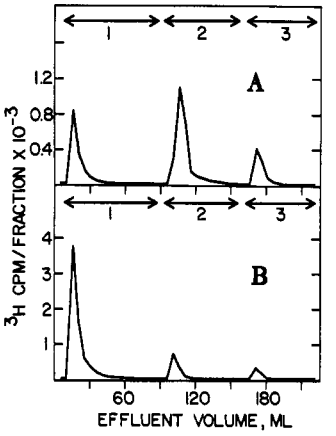

FIGURE 5: Cellulose column radioactivity elution profile for [^{3}H]-RNA digested with ribonuclease prior to dodecyl sulfate–phenol extraction as described in the text. (A) Embryos labeled for 30 min; (B) embryos labeled for 240 min.

proportion of ds-RNA. Material eluting in the third chromatographic peak was also characterized by its melting temperature in 0.1 × SSC (Figure 6). The thermal stabilities of ds-RNA purified before and after annealing are indistinguishable. There appear to be some approximately complementary and moderately rapidly annealing RNA sequences in sea urchin embryos which are not initially duplexed but which can be duplexed under annealing conditions.

Approximately 0.2% of the total labeled RNA is double stranded after a 30-min pulse while 0.06% is double stranded after a 240-min pulse. Since the absolute amount of RNA accumulated during a given pulse and the rate of turnover of the RNA is known in sea urchin embryos (Brandhorst and Humphreys, 1971), these percentages can be used to calculate the rates of synthesis and turnover of ds-RNA. Clearly this RNA must turn over more rapidly than the bulk of labeled RNA molecules, *i.e.*, mRNA. The kinetics of accumulation suggest that the half-life of the ds-RNA is similar to that for nuclear RNA which turns over in the nucleus with a half-life of approximately 7 min. Brandhorst and Humphreys (1972) showed that under conditions of steady-state labeling of RNA approximately one-third of the labeled RNA is nuclear while the remainder is cytoplasmic (cytoplasmic half-life of about 60 min). Since under steady-state labeling conditions only about one-third the fraction of labeled RNA as for a short pulse is double stranded, the ds-RNA behaves kinetically as nuclear RNA. Assuming the absolute rate of synthesis of nuclear RNA is 9.7×10^{-15} g nucleus^{-1} min^{-1} (Brandhorst and Humphreys, 1971) then ds-RNA is synthesized in sea urchin blastulae at a rate of approximately 2×10^{-17} g nucleus^{-1} min^{-1}.

DNA–RNA hybrids do not survive the nuclease digestions employed here (Nygaard and Hall, 1964). However, because ds-RNA represents a very small portion of the nucleic acids, the possibility that the third chromatographic peak represents a small fraction of DNA–RNA hybrids uniquely stable to the isolation procedure was examined. Embryo DNA was uniformly labeled to high specific activity by supplying embryos with [5-^{3}H]thymidine (Schwarz, 6 Ci/mmole) in 25-μCi/ml doses at 0.5-, 4.5-, 7.5-, 9.5-, and 11.5-hr postfertilization. Embryos were then extracted, digested, and chromatographed and the third chromatographic fraction was assayed for tritium; 5.1×10^4 cpm of the 1.7×10^7 cpm incorporated into nucleic acids chromatographed with the third peak, but only 14 cpm of this was alkali stable. The number of nuclei per blastula is approximately 450, there is 1.6×10^{-12} g of DNA nucleus^{-1} and 1.5×10^6 embryos were used in this experiment; hence 14 cpm represents 7.4×10^{-10} g of DNA. By comparison 1.5×10^6 embryos would yield approximately 10^{-7} g of ds-RNA. Thus on a mass basis DNA is less than 1% of RNA in the third peak and it is evident that DNA–RNA hybrids do not significantly contribute to ribonuclease-resistant RNA chromatographing in the third peak.

The rate of synthesis of 18S and 28S rRNA in sea urchin blastulae is 1.2×10^{-17} g nucleus^{-1} min^{-1} compared to 2×10^{-17} g nucleus^{-1} min^{-1} for ds-RNA. Assuming liberally that 10% of newly synthesized rRNA is ribonuclease resistant it is obvious that the low levels of ds-RNA observed in sea urchin embryos do not represent degradation products of newly synthesized rRNA.

Site of Synthesis of ds-RNA. Embryo ds-RNA could represent a product of mitochondrial RNA metabolism. This can readily be tested in sea urchin embryos since immediately after fertilization most RNA synthesis is mitochondrial (Chamberlain, 1968; Craig, 1970). Fertilized eggs were incubated with 10 μCi/ml of [^{3}H]uridine from 10- to 100-min postfertilization and ds-RNA was isolated. Labeled ds-RNA was

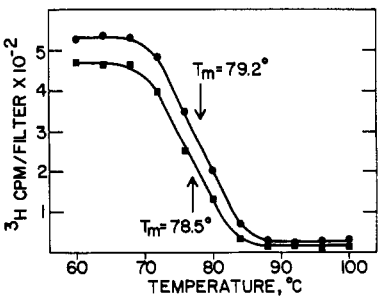

FIGURE 6: Melting curves in 0.1 × SSC of ds-[^{3}H]RNA purified before (■) or after (●) annealing of total nucleic acid.

TABLE IV: Purification of ds-RNA from Nuclei and Cytoplasm.

	Duration of Pulse	
	8 min	120 min
Per cent labeled RNA		
Nuclei	81	41
Cytoplasm	19	59
Per cent labeled RNA double stranded		
Nuclei	0.32	0.33
Cytoplasm	0.14	0.095
Per cent labeled ds-RNA		
Nuclei	81	66
Cytoplasm	19	34
Per cent total DNA radioactivity in cytoplasm	13	10

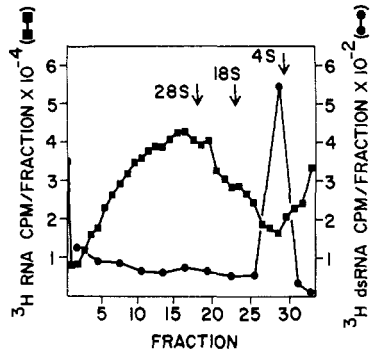

FIGURE 8: Resolution of [^{3}H]RNA (15–35% sucrose gradients, 12 hr at 25,000 rpm in the SW-27 rotor) prior to purification of ds-RNA. 9-drop fractions from parallel gradients were used to determine total and alkali-stable radioactivity. 27-drop fractions from a third gradient were digested and chromatographed to assay ds-[^{3}H]RNA. (■) Total RNA radioactivity; (●) ds-[^{3}H]RNA.

less than 0.004% of total labeled RNA and thus embryo ds-RNA is probably not of mitochondrial origin.

Since ds-RNA accumulates similarly to nuclear RNA, the site of synthesis and turnover of ds-RNA was investigated by fractionating the cells into nuclei and cytoplasm before extracting RNA. A culture of embryos was labeled from 30- to 90-min postfertilization with 1 μCi/ml of [^{14}C]thymidine (Schwarz, 42.0 mCi/mmole) in order to label DNA as a nuclear marker. At 15-hr postfertilization half of the culture was labeled with 50 μCi/ml of [^{3}H]uridine for 8 min while the other half was labeled with 10 μCi/ml of [^{3}H]uridine for 120 min. Following labeling the embryos were chilled and fractionated as described in the Experimental Section. Each fraction was extracted and the ds-RNA purified (Table IV). Most of the ds-RNA is in the nuclei after both 8- and 120-min labeling. The ^{14}C radioactivity indicates that nuclear contamination of cytoplasm in these experiments amounts to 10–20%. When this contamination is subtracted virtually all of the ds-RNA appears to be confined to the nuclei.

Although the preceding experiment does not suggest that ds-RNA is cytoplasmic this was examined more carefully. The major class of cytoplasmic RNA which becomes labeled in 15-hr embryos is mRNA associated with the polysomes (Brandhorst and Humphreys, 1972). Embryos were incubated for 6 hr with 10 μCi/ml of [^{3}H]uridine and polysomes prepared as described in the Experimental Section. These homogenates along with controls for nuclear contamination which contained 50 mM EDTA (Penman *et al.*, 1968) were layered on sucrose gradients and centrifuged. The gradients were separated into material sedimenting faster than monoribosomes or slower than and including monoribosomes and each part assayed for ds-RNA. There was no detectable ds-RNA associated with the polysomes, further confirming ds-RNA is not associated with cytoplasmic structures.

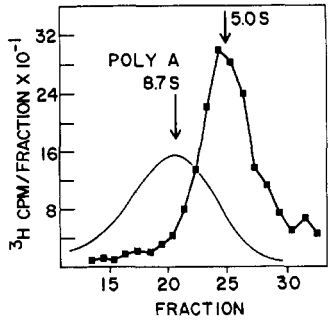

FIGURE 7: Sedimentation profile of purified ds-[^{3}H]RNA centrifuged on a 5–25% sucrose gradient 18 hr at 25,000 rpm in the SW-27 rotor. 9-drop fractions were assayed for acid-precipitable radioactivity (■). Polyriboadenylate (—) was included as an internal marker and calibrated in parallel gradients with reference to 18S and 28S rRNA.

Base Composition of ds-RNA. The base composition of ds-RNA was determined using embryos labeled with 33 μCi/ml of [^{32}P]P_i (New England Nuclear, carrier free) for 15 hr beginning at 15-min postfertilization, or with 500 μCi/ml of [^{32}P]P_i for 15 min beginning at 15-hr postfertilization (Table V). These analyses showed a high adenine content unmatched by equal uracil content and suggest that polyadenylate might be associated with the ds-RNA. Since polyadenylate sequences would not be digested by pancreatic and T1 ribonucleases and since polyadenylate does not cochromatograph with ds-RNA on the cellulose column (Figure 1A), the possibility that ds-RNA is bound to polyadenylate was examined. One-half of the ds-RNA synthesized during the 15-hr pulse was heat denatured, diluted with an equal volume of water, and digested 30 min at 37° with 10 μg/ml of pancreatic RNase and 20 unit/ml of T1 RNase, and the acid-soluble and acid-precipitable fractions analyzed for base composition. Approximately 35% of the ribonuclease-resistant material labeled with ^{32}P and chromatographing as ds-RNA appears to be polyadenylate. All other material becomes ribonuclease sensitive after denaturation. The base composition of the latter material is that expected of a double-helical polynucleotide.

Sedimentation of ds-RNA. ds-RNA purified on the cellulose column was centrifuged on 16-ml 5–25% (w/v) linear sucrose gradients in dodecyl sulfate buffer (0.10 M NaCl, 1 mM Na_2-EDTA, 10 mM Tris-HCl, and 0.5% dodecyl sulfate, pH 7.50) 18 hr at 25° at 25,000 rpm in the SW-27 rotor. Polyadenylic acid which is not affected by contaminating ribonuclease activities, was used as an internal marker of about 9 S. The sedimentation value of the polyadenylate was determined by sedimentation with sea urchin 18S and 28S rRNAs. The ds-RNA sediments at 4–6 S regardless of the duration of radioactive labeling (Figure 7).

ds-RNA is prepared using extensive nuclease digestion which may release these 4- to 6S pieces from larger molecules.

TABLE V: Base Composition of Ribonuclease-Resistant RNA.

	Mole %			
	A	C	G	U
Third chromatographic peak, 15-min pulse	40.0 ± 0.6	19.2 ± 0.3	21.8 ± 0.3	19.0 ± 0.3
Same, 15-hr pulse	58.0 ± 0.3	14.7 ± 0.4	14.8 ± 0.4	12.5 ± 0.1
Low-salt digest, supernatant	23.1 ± 0.2	27.0 ± 0.2	27.1 ± 0.2	22.8 ± 0.2
Low-salt digest, acid insoluble	96.6 ± 0.5	1.4 ± 0.1	1.2 ± 0.1	0.8 ± 0.05

This was examined using 15-hr embryos which were labeled for 60 min with 25 μCi/ml of [^{3}H]uridine. Intact RNA was prepared according to Emerson and Humphreys (1970) and centrifuged on three 15–35% linear sucrose gradients in dodecyl sulfate buffer for 12 hr at 25° at 25,000 rpm in the SW-27 rotor. One gradient was fractionated as indicated in Figure 8. The resulting fractions were precipitated with ethanol and then assayed for ds-RNA content using nuclease digestion and the cellulose column. The other gradients were assayed directly for total and alkali-stable, acid-insoluble radioactivity (Figure 8). ds-RNA can be isolated from molecules distributed throughout the gradient. It seems probable that a significant fraction of the ds-RNA extracted from sea urchin embryos exists *in vivo* as portions of larger molecules. However, under conditions in which very large RNA molecules could be resolved, approximately half of the ds-RNA is of the same size as the ds-RNA produced by ribonuclease treatment.

Discussion

Metabolism of complementary RNA sequences and ds-RNA is well described in replication of riboviruses (Spiegelman *et al.*, 1968; Baltimore, 1971). At present there is no evidence for RNA-dependent RNA synthesis in uninfected animal cells, although labeling of ds-RNA seems less sensitive to Actinomycin D than is labeling of total RNA (Montagnier, 1968; Stern and Friedman, 1970, 1971). The mechanism of production of ds-RNA in eukaryotic cells has not been established. It seems clear that production of virus-specific ds-RNA throughout vaccinia infection is a DNA-dependent synthesis (Colby *et al.*, 1971). It has been suggested that ds-RNA arises from overlapping transcription of antipolar genes in viral systems (Bøvre and Szybalski, 1969; Jurale *et al.*, 1970). Stern and Friedman (1971) hybridized chick embryo fibroblast ds-RNA to chick liver DNA, but only a small fraction of the input ds-RNA hybridized under the conditions employed. Montagnier and Harel (1971) hybridized rat liver ds-RNA to rat liver DNA; the results suggest that ds-RNA is homologous with rather highly reiterated DNA sequences but do not establish DNA-dependent synthesis of ds-RNA. Our procedures do not significantly anneal complementary single strands but such sequences evidently are present in embryos and ds-RNA *can* arise *via* annealing of these. The possibility exists that the double-stranded conformation is produced *via* post-transcriptional annealing in the cell of complementary, highly redundant RNA sequences.

Polyriboadenylate metabolism and ATP:RNA adenylyl transferases have been reported in prokaryotic and eukaryotic systems (for references, see Twu and Bretthauer, 1971) including sea urchin embryos (Hyatt, 1967). Sequences rich in adenylate occur in mRNA (Lim and Canellakis, 1970; Lee *et al.*, 1971) and in nuclear and rapidly labeled polysomal RNA in HeLa cells (Edmonds *et al.*, 1971; Darnell *et al.*, 1971). The significance of an association between polyadenylate and ds-RNA in sea urchin embryos is not known. There is increasing evidence that posttranscriptional polyadenylylation is required for stabilization of nuclear mRNA sequences (Philipson *et al.*, 1971). However, we have no evidence that ds-RNA-bound polyadenylate in sea urchin embryos shares a common origin or a precursor relationship with cytoplasmic polyadenylate sequences.

With short labeling times in a variety of cells, characteristically a few tenths of a per cent of the labeled RNA is double stranded (Montagnier, 1968). With longer labeling times this percentage is decreased by up to an order of magnitude. We have shown that ds-RNA is synthesized in sea urchin blastulae at a rate of 2×10^{-17} g nucleus^{-1} min^{-1} or approximately 0.2% of the rate of synthesis of nuclear RNA. The ds-RNA cannot consist of ribonuclease-resistant portions of rRNA and turns over more rapidly than the majority of newly synthesized RNA molecules. The kinetics of accumulation of ds-RNA are quantitatively accounted for by assuming that ds-RNA represents a subclass of rapidly turning-over nuclear RNA sequences. In accord with this interpretation are the following observations: (i) ds-RNA is synthesized in and probably confined to the nucleus; (ii) the percentage of nuclear RNA which is double stranded does not decrease with increasing labeling times (Table IV); (iii) ds-RNA exists *in vivo* both as portions of much larger ribonuclease-sensitive molecules and as 4- to 6S species similar in size to the ds-RNA prepared using ribonuclease. Taken together with the suggestions that ds-RNA is bound to polyadenylate and hybridizes with highly reiterated DNA sequences, we propose that cellular ds-RNA is transcribed as heterogeneous nuclear RNA in concert with messenger sequences and that the duplex RNA represents a portion of the transcript which is degraded in the nucleus in conjunction with the processing of mRNA and its transport to the cytoplasm.

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In Vitro Transcription of Mitochondrial Deoxyribonucleic Acid from Yeast†

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ABSTRACT: Mitochondrial and nuclear DNAs from various wild-type and petite yeast strains have been highly purified by hydroxylapatite chromatography. Yeast mitochondrial, nuclear, and *Escherichia coli* DNA-dependent RNA polymerases were used to transcribe mitochondrial DNA of yeast into RNA. The RNA produced by these enzymes was used to study both the nature of the enzyme products and the properties of the template DNAs. The *E. coli* enzyme and mitochondrial enzyme transcribed the ribosomal genes of mitochondrial DNA as shown by competition with cold mitochondrial ribosomal RNA. The labeled *in vitro* synthesized RNAs were hybridized to various DNAs in an attempt to compare the extent of homology among these DNAs. Very little homology was indicated between nuclear and mitochondrial DNA of yeast. Total cell DNA from two petite mutants lacking the mitochondrial satellite band showed the same low degree of homology to mitochondrial DNA. Wild-type mitochondrial DNAs differed in their homologies to each other and appeared to contain different amounts of repetitive DNA. Mitochondrial DNAs from two petite mutants showed reduced homology to wild-type mitochondrial DNA. However, no additional sequences which differed from those of wild-type mitochondrial DNA could be detected in the mitochondrial DNA of one petite mutant studied, thus indicating a simple deletion mechanism for the origin of this strain. This petite mutant lacks 50–60% of the wild-type mitochondrial genome transcribed *in vitro*.

The interest in the genetic function of mitochondrial DNA has initiated during the last few years a large number of investigations on this subject (Borst and Kroon, 1969; Ashwell and Work, 1970). Yeast mitochondrial DNA is particularly interesting as mutations in this DNA can block mitochondrial functions but are not lethal to the cell. Very often mutations cause a change in the buoyant density of mitochondrial DNA (Mounolou *et al.*, 1966; Bernardi *et al.*, 1968; Mehrotra and Mahler, 1968) and even a complete loss of mitochondrial DNA in this organism has been reported (Goldring *et al.*, 1970; Nagley and Linnane, 1970; Michaelis *et al.*, 1971). Relatively large amounts of yeast mitochondrial DNA can be isolated by chromatography on hydroxylapatite (Bernardi *et al.*, 1970). This enabled us to set up and characterize an *in vitro* transcription system using yeast mitochondrial DNA as template. We have used this system for studying the question of sequence homology be-

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