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Synthesis and Decay Rates of Major Classes of Deoxyribonucleic Acid Like Ribonucleic Acid in Sea Urchin Embryos*

Bruce P. Brandhorst† and Tom Humphreys

ABSTRACT: A method is described for measuring the instantaneous rates of synthesis and the spectrum of stabilities of unstable RNA by analyzing the kinetics of entry of radioactive adenosine into ATP and into RNA. This method was applied to sea urchin embryos during their development when the level of accumulation of heterogeneous, unstable RNA with DNA-like base compositions decreases substantially. The RNA appeared to fall into two classes determined on the basis of stability. One-third of the steady-state level of unstable RNA has a half-life of 5–10 min and the rest a half-life of 60–90 min.

This spectrum does not change between blastula and pluteus stages, while the instantaneous rate of synthesis of RNA per nucleus is decreasing.

Recent experiments have shown that the accumulation per nucleus of DNA-like RNA[1] decreases gradually during the development of sea urchin embryos from cleavage to pluteus stages (Emerson and Humphreys, 1970). Previous studies indicate that most DNA-like RNA synthesized in animal cells is unstable; most of it appears to be restricted to the nucleus and decays with an average half-life estimated at from 3 to 30 min (Attardi *et al.*, 1966; Soiero *et al.*, 1968; Scherrer *et al.*, 1966; Penman *et al.*, 1968). A small fraction associated with polysomes has an estimated half-life of several hours (Penman *et al.*, 1963; Attardi *et al.*, 1966; Penman *et al.*, 1968). The extent of heterogeneity in the stabilities of these two classes of RNA and the possibility of other classes of unstable RNA (Penman *et al.*, 1968) have not been scrutinized. Because most or all DNA-like RNA is unstable, its level of accumulation is related to both its rates of synthesis and its rates of decay. In order to analyze the developmental changes in levels of accumulation, we had to determine the instantaneous rates of synthesis and the spectrum of stabilities of all DNA-like RNA molecules synthesized in the embryos. This was done by determining the kinetics of entry of radioactive adenosine into the ATP precursor pool and into RNA. Sea urchin embryos were favorable material for the study of the complete spectrum of unstable RNA species because unstable RNA represented at least 85% of the newly synthesized RNA accumulated during several hours of labeling (Emerson and Humphreys, 1970; and Results).

Material and Methods

Culturing and Processing of Embryos. Embryos of *Lytechinus pictus* were cultured at 16.5° on a rotary shaker as an 0.5% suspension (v/v) in artificial sea water, with penicillin (80 unit/ml) and streptomycin (50 μg/ml). Embryos cultured beyond mesenchyme blastula stage were resuspended at 18 and 40 hr in fresh sea water at a concentration of 0.05% embryos. The experiments were performed on mesenchyme blastulae (18–22.5 hr after fertilization) or early plutei (44–50 hr). Cultures used had at least 98% fertilization and normal development.

Blastulae were suspended at 2.5% concentration, and incubated with 10 μCi/ml of [8-^{3}H]adenosine (28 Ci/mmole; Schwartz). Sample aliquots of 0.8 ml were taken at various times after addition of the radioactive label. Plutei, suspended at 0.5%, were incubated with 2.5 μCi/ml of tritiated adenosine; 4-ml samples were taken. The sample of embryos was squirted into excess sea water cooled on ice and washed twice through a 3:1 mixture of acid sea water (0.02 M acetate, pH 4.5) and isotonic sucrose to remove external isotope. The number of embryos per sample (about 20,000) was determined by the method of Hinegardner (1967). The number of nuclei at the beginning and end of each period of incubation was determined by counting stained nuclei in fixed preparations (Emerson and Humphreys, 1970). The pelleted embryos were suspended in ice-cold 0.5 N perchloric acid (always used cold), homogenized with 25 strokes of tight-fitting Dounce homogenizer (Kontes Glass Co.), cooled on ice for 15 min, and centrifuged for 10 min at 27,000*g*. The supernatant was used immediately for isolation of nucleotides, and the pellet resuspended in 2 ml of 0.5 N perchloric acid by vigorously squirting it through an 18-gauge syringe needle, and recentrifuged. The pellet was washed 4 more times with perchloric acid and used to determine radioactivity in RNA.

Counting of Radioactivity. Samples solubilized in 0.3 N

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[1] DNA-like RNA has a base composition similar to DNA and is heterogeneous in size (Emerson and Humphreys, 1970).

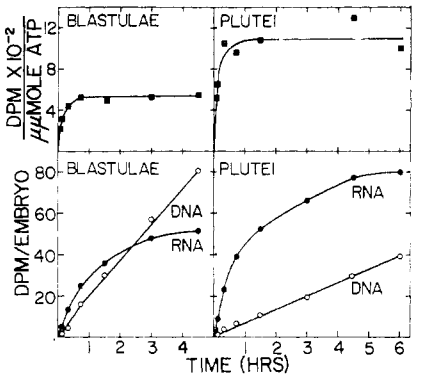

FIGURE 1: Kinetics of incorporation of [8-³H]adenosine into ATP, RNA, and DNA of mesenchyme blastulae and early plutei.

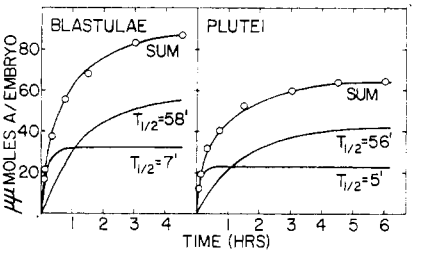

FIGURE 2: Molar accumulation of newly synthesized RNA. Circles show accumulation of adenosine in RNA calculated from the incorporation data in Figure 1 as described in the text. Smooth lines indicate the kinetics of accumulation of adenosine in RNA according to the model presented in the text.

NaOH were dissolved in toluene scintillation fluid (0.4% 2,5-diphenyloxazole, 0.005% 1,4-bis[2-(5-phenyloxazolyl)]-benzene) with an appropriate concentration of Beckman Bio-Solve solubilizers. Scintillation counting was done in a Beckman LS-200 to an accuracy of 2%. All samples were corrected for quenching by the external standard method.

Determination of the Specific Activity of ATP. A detailed description and justification of the procedures used is forthcoming (Emerson and Humphreys, 1971). The total nucleotides in the perchloric acid supernatant were bound to activated charcoal, which was then washed 4 times with water. The nucleotides were eluted by incubation at 37° in 0.1 N NH_4OH in 50% ethanol. The eluate was dried, redissolved in 0.1 N NH_4OH, and spotted on an Eastman Chromagram cellulose thin-layer chromatography plate. The nucleotides were separated by ascending chromatography in a system of 66:33:1 isobutyric acid–water–NH_4OH, respectively. This procedure resolves ATP from dATP, ADP, and AMP, which contain most of the radioactivity not in ATP. The ATP spot was cut out and eluted in 0.04 M glycylglycine buffer, pH 7.4. The amount of ATP in a small aliquot in a scintillation vial was determined by the luciferin-luciferase assay (McElroy, 1947; Cole *et al.*, 1967) employing a Beckman scintillation counter. Firefly lantern extract was obtained from Sigma Chemical Co. and made up according to their instructions. ATP (Calbiochem) was used to standardize the assay. The assayed sample was dissolved in scintillation fluid and counted for radioactivity. The specific radioactivity of the ATP was calculated as disintegrations per minute (dpm) per mole.

Determination of the Radioactivity in RNA and DNA. The perchloric acid pellet was hydrolyzed for 90 min at 37° in 0.3 N NaOH (Fleck and Munro, 1962). The sample was cooled and the unhydrolyzed DNA precipitated from an aliquot by acidification with perchloric acid and collected on a Millipore filter. Another aliquot was precipitated with perchloric acid and the supernatant collected after centrifugation. The radioactivity and absorbance at 260 mμ (OD_{260}) of the supernatant were determined and a value for specific activity of RNA as acid-precipitable, alkaline-labile radioactivity per OD_{260} unit calculated.

The tritium of adenine exchanges with water in base (Wilt, 1969). This exchange was measured by binding DNA and RNA oligonucleotides in an aliquot of the NaOH hydrolysate to Dowex 1-X8 formate. The unbound radioactivity was the exchanged tritium which was generally about 6% of the total acid-precipitable radioactivity. The radioactivity in RNA and DNA was calculated assuming that the exchange per tritium atom was the same in RNA and DNA.

The GTP precursor pool also becomes radioactive because of purine interconversions. Since the specific activity of only the ATP pool was determined, the radioactivity in GMP of RNA was measured and subtracted from the total radioactivity in RNA. The RNA was completely hydrolyzed to 2′(3′)-ribonucleoside monophosphates by an 18-hr incubation in 0.3 N NaOH, and the nucleotides separated on thin-layer plates as for ATP. Radioactivity appeared in only GMP and AMP spots. About 40% of the tritium of G exchanges with water and 65% of that of A after 18-hr hydrolysis (B. P. Brandhorst, unpublished observations). The radioactivity in the two nucleotides was corrected for these different rates of exchange. This procedure also subtracts any ATP which is trapped in the acid precipitate. This ATP, which may be significant after short labels, is stable during the alkaline hydrolysis and chromatographs with GMP. Since ATP exchanges tritium with water at a different rate than GMP, this may introduce an error because the radioactivity in the GMP spot was assumed to exchange as G. Procedures which resolve ATP and GMP (C. P. Emerson and T. Humphyreys, in preparation) indicate that the error introduced could not exceed 6% of the radioactivity in RNA of any sample reported here and that it is much less in most samples. Having made the corrections described, a value for the disintegrations per minute of tritiated AMP incorporated into RNA per OD_{260} (*i.e.*, per embryo) was calculated for each sample. Usually about 90% of the acid-precipitable, alkaline-labile radioactivity is in the AMP of RNA.

Sucrose Gradient Analysis of RNA. RNA was purified using a cold phenol extraction followed by a hot extraction (Emerson and Humphyreys, 1970). It was sedimented through a 38-ml 15–35% sucrose gradient at 26,000 rpm for 9 hr at 25° in a Beckman SW27 rotor. Acid-precipitable, alkaline-labile radioactivity for each fraction was determined.

Results

The entry of tritiated adenosine into the ATP, RNA, and DNA of mesenchyme blastulae and early plutei from the same female is shown in Figure 1. The rate of accumulation of radioactive DNA is constant, while the rate of RNA accumulation gradually decreases with time even though the specific activity of the ATP does not fall. This indicates that the DNA is stable and the bulk of the radioactive RNA is unstable.

The actual amount of RNA represented by the radioactivity in RNA was calculated (circles in Figure 2). The

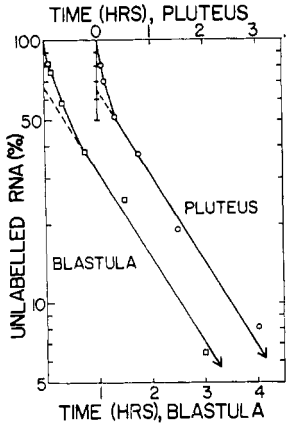

FIGURE 3: Standard decay analysis of accumulation of radioactive RNA (replotted from Figure 2). The logarithm of the percentage of total unstable RNA remaining unlabeled is plotted against time. The time scale for the pluteus is shifted 1 hr to the right for comparison. The slopes of the lines are proportional to the decay constants of the RNA. Extrapolation of the latter part of the decay curve to time 0 (broken lines) determines the relative steady-state level of the more stable RNA.

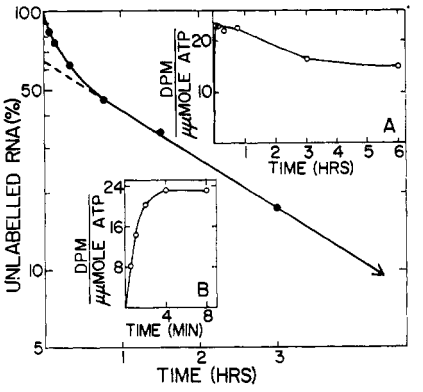

FIGURE 4: Decay analysis of accumulation of RNA in embryos after rapid equilibration of the ATP pool. Mesenchyme blastulae in a 10% suspension were incubated with 10 μCi/ml of [^{3}H]adenosine for 3 min and then diluted to a 1% suspension. In order to show the complete curve of radioactivity into ATP, the data are plotted on different scales in insets A and B.

radioactivity in RNA at time t was divided by the average specific activity of the ATP during the incubation time over which the RNA had accumulated. The average specific activity of ATP was calculated by integrating the ATP specific activity curve from time 0 to time t and dividing by t. This operation gives a value for the amount of AMP in RNA which has accumulated since the addition of isotope and would be entirely accurate if the RNA were stable and there were no significant compartmentalization of the ATP pool such that the specific activity for the total ATP pool was that of the RNA precursor pool at all times. These conditions will be examined more carefully later. The data show that the RNA accumulates rapidly at first but gradually approaches a maximum level.

If an equilibrium situation exists in which the rate of synthesis of each class of RNA molecules is equal to its rate of degradation, the accumulation curve of radioactively labeled RNA in Figure 2 would be the inverted curve for decay of unlabeled molecules beginning at the time of addition of isotope. After the addition of isotope, every molecule synthesized would be labeled and whenever an unlabeled molecule decayed, it would be replaced by a labeled RNA molecule. The decay of a radioactive molecule would not be an observable event since accumulation is corrected for the changing average specific activity of the ATP pool. If decay follows first-order kinetics, the curve for the decay of unlabeled molecules can be subjected to standard decay analysis as in Figure 3. The foregoing analysis is formally justified in the Appendix, which is based on equations derived by Fritz *et al.* (1969). If all the RNA had a uniform stability, the curves in Figure 3 would approximate straight lines with slopes directly proportional to the decay constants. They do not, indicating the presence of two or more classes of RNA with different stabilities.

The simplest assumption which fits the data is that there are two classes of RNA with different stabilities. Model curves (continuous lines in Figure 2) showing the accumulation of these two classes can be derived from graphical analysis of the curves in Figure 3. The total curve, which is the sum of the two model curves with single decay rates, fits the observed values (circles) very well. The parameters for these model curves indicate that about 35% of the steady-state level of newly synthesized RNA decays with a half-life of 5 min in the pluteus and 7 min in the blastula. The remaining 65% decays with a half-life of about 1 hr in embryos of both stages. Models with more than two classes of RNA could also be fitted to the experimental data but no more accurately. The two curves do define the approximate limits of stabilities of major fractions of the RNA in these cells.

The instantaneous rate of synthesis for each class of RNA is assumed to be equal to the instantaneous rate of decay which can be calculated as the product of the decay constant times the steady-state level of the class of RNA. The sum of the instantaneous rates of synthesis from the model curves in Figure 2 for the mesenchyme blastulae is 3.9×10^{-15} mole of A/embryo per min. About 85% of the synthesis is due to the very unstable RNA and 15% is due to the more stable RNA. Using a base composition of 30% A (Emerson and Humphreys, 1970) and 470 nuclei per embryo, this instantaneous rate of synthesis equals 9.7×10^{-15} g of RNA/nucleus per min. This value for early pluteus with 1340 nuclei is 3.1×10^{-15} g/nucleus per min.

These calculations are based on the conditions that the ATP precursor pool is not compartmentalized and that the half-life of the RNA is not short compared to the time for the pool to equilibrate. It was not possible to evaluate these conditions by independent experiments. However, the results obtained when the ATP pool equilibrates at different rates might be different if these conditions were violated. Figure 4 shows an experiment in which the maximum specific radioactivity of ATP was reached by 4 min rather than by 30–40 min as in the other experiments described. Again, by the simplest interpretation, one-third of the RNA turns over with a half-life of 7 min and the remainder with a half-life of 82 min.

Since the rapidly decaying RNA predominates after short incubations with isotope and the more stable RNA predominates later, the size distributions of these two classes of RNA were compared. The RNA of mesenchyme blastulae was labeled for 5 min and for 6 hr with [^{3}H]adenosine, extracted, and fractionated on sucrose gradients (Figure 5). In both cases the predominant RNA is very high molecular weight with a peak at 34 S. There is no major difference in size distribution, although there is relatively more 4–5S RNA after longer labeling periods.

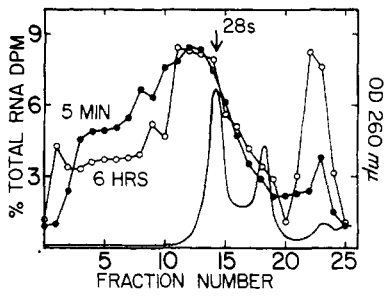

FIGURE 5: Sucrose gradient analysis of RNA extracted from mesenchyme blastulae labeled with tritiated adenosine for 5 min or 6 hr. The labeling conditions are described in Methods. The percentage of the total radioactivity sedimenting greater than 10 S is plotted for each fraction. Solid line shows OD_{260}.

Discussion

For this analysis of RNA synthesis we assumed that the measured specific activity of the total ATP pool is that of the RNA precursor pool. All evidence is consistent with this assumption. There does not seem to be any transient compartmentalization, since the results were not affected by different kinetics of entry of radioactivity into the ATP pool. Similar experiments measuring incorporation of radioactive guanosine (Kijima and Wilt, 1969) or uridine (Roeder and Rutter, 1970) into the nucleoside triphosphate pools and RNA gave graphically determined instantaneous rates of synthesis of RNA similar to the ones calculated from our results with radioactive adenosine. It would be surprising if the ATP, GTP, and UTP pools, which are very different in size and function were similarly compartmentalized. The specific radioactivity of DNA and the amount of newly synthesized DNA accumulated as determined by the increase in the number of nuclei per embryo during the course of the labeling were used to calculate the average specific radioactivity of the dATP precursor pool. This value is the same as the specific radioactivity of the total ATP pool, indicating that the total ATP and dATP precursor pools are equilibrated; this is further evidence for the validity of the specific radioactivity values used for the RNA precursor ATP pool.

We assumed that decay followed first-order reaction kinetics. The most likely alternative is a population of molecules having a spectrum of finite lifetimes. Curves in Figures 1, 2, and 3 could easily be accounted for by this possibility. The exponential decay of radioactive label after inhibition of RNA synthesis (Levinthal *et al.*, 1962) is also consistent with both possibilities. Any single species of RNA molecule, though, should decay exponentially if degradation is a first-order reaction, or linearly if it has a finite lifetime. An exponential decay of the capacity to synthesize a specific enzyme in the absence of RNA synthesis has been observed in bacteria (Hartwell and Magasanik, 1963) and rat liver (Tschudy *et al.*, 1965), indicating that some defined species of RNA do decay with apparent first-order kinetics.

Our analysis of the decay of RNA depends on the existence of a steady-state situation in which the rate of synthesis of each class of RNA molecules is equal to its rate of decay. The observed steady-state equilibrium of radioactivity with RNA and constant amount of RNA per embryo are consistent with this assumption. Although there is a small percentage of accumulation of stable RNA species (Emerson and Humphreys, 1970) it is less than the experimental error of about 15% and would not significantly alter our analysis if it accounted for 15% of the radioactivity incorporated after a 6-hr incubation.

Most attempts to determine the stability of RNA in animal cells have been based on the decay kinetics of radioactively labeled RNA after the inhibition of RNA synthesis with a drug such as actinomycin D. Such experiments are subject to uncertainty about the total physiological effects of the drug. An average half-life of 3 min has been calculated for nuclear RNA in HeLa cells under normal growth conditions at 37° (Soeiro *et al.*, 1968). This was calculated from the relative rates of synthesis and accumulation of ribosomal and DNA-like RNA. The same data also show that the instantaneous rate of synthesis of nuclear RNA is many times that of polysomal RNA. That analysis gave an average half-life of nuclear RNA and thus did not provide information on the spectrum of stabilities of the RNA. Other data suggest that polysomal RNA has a half-life of about 3 hr (Penman *et al.*, 1963). The two classes of heterogeneous RNA with different stabilities in sea urchins may be equivalent to the nuclear and polysomal RNA of HeLa cells. Our experiments in progress on the accumulation of RNA in nuclei and cytoplasm are consistent with this hypothesis (Brandhorst, 1970).

The decrease in accumulation of DNA-like RNA during development is entirely due to a decrease in the instantaneous rate of synthesis per nucleus. This decrease has been observed previously (Kijima and Wilt, 1969; Roeder and Rutter, 1970). The synthesis of all classes of RNA appears to be similarly reduced. This is consistent with the decreasing activities of nucleoplasmic RNA polymerase bound to DNA in nuclei of sea urchin embryos reported by Roeder and Rutter (1970). The relationship of qualitative changes in synthesis of specific RNA molecules during development to these quantitative changes are unknown.

Acknowledgment

We wish to thank Dr. Charles P. Emerson for many helpful discussions.

Appendix

Making the same assumptions as ours, Fritz *et al.* (1969) derived the following equation for a class of molecules which have the same stability (appropriate changes in notation have been made): $\ln(R_{max}^* - R^*) = -V_s t/R - \ln(R_{max}^* - R_0^*)$, where V_s is the instantaneous rate of synthesis in moles of adenosine/min per embryo; R^* is the radioactivity in dpm/embryo of adenosine in that class of RNA; $R_0^* = R^*$ at time $t = 0$ when the specific activity of the ATP pool has reached a constant maximum (A_{max}^*); and R = moles of adenosine in that class of RNA per embryo. A plot of $\ln(R_{max}^* - R^*)$ *vs.* time should be a straight line with a slope of $-V_s/R$; the rate constant for decay is V_s/R, while V_s is the rate constant for synthesis. An analysis of the data in Figure 1 by this procedure indicated that much of the RNA turns over with a half-life of about an hour; extrapolation of this curve to time 0 indicated the presence of some less stable RNA.

It is desirable to have immediate equilibration of the ATP pool in order to analyze the kinetics of accumulation of all the radioactive RNA by the above calculation. An operational approximation to this situation was made by multiplying the molar amounts of RNA calculated for Figure 2 by A_{max}^*. This results in an approximate value of R^* which would have been observed had the ATP pool equilibrated immediately.

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Histone Synthesis. Assignment to a Special Class of Polyribosomes in Sea Urchin Embryos*

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ABSTRACT: In the early sea urchin blastula the s-polysomes (3 to 7 ribosomal aggregates) constitute more than 70% of the polysomal ribosomes. The function of this predominant class of polyribosomes was pursued through an examination of associated nascent proteins. Histone-like proteins were purified from the nascent proteins of s-polysomes by successive acid extraction, cation-exchange chromatography, and acrylamide gel electrophoresis. A similar procedure with the nascent proteins from the larger polyribosomes yielded proteins that did not appreciably resemble histones. We conclude that the s-polysomes are the site of chromosomal histone synthesis. The nascent proteins of these polyribosomes have a substantially enhanced incorporation of arginine and lysine relative to tryptophan, as compared to these relative incorporations in the larger polyribosomes. This enhancement reflects the unique overall amino acid composition of histones.

The extent of this enhancement changes during embryonic development in a manner parallel to changes in DNA synthesis.

A class of polyribosomes containing 3–7 ribosomes/mRNA plays a prominent role in the early development of the sea urchin embryo (Infante and Nemer, 1967). These polyribosomes, designated "s-polysomes," increase in concentration from barely perceptible amounts in the early cleaving embryo to over one-third of the total ribosomes in the 10-hr, 200-cell early blastula of *Stronglyocentrotus purpuratus*. The formation of s-polysomes is largely dependent on RNA newly synthesized by the early stage embryo, rather than upon RNA preexisting in the egg (Infante and Nemer, 1967). This new polysomal RNA is predominantly of a 9–10S class (Nemer and Infante, 1965; Kedes and Gross, 1969). The significance of the very extensive synthesis of this narrow class of mRNAs and the accumulation of these polyribosomes in early sea urchin blastulae has been the subject of several recent studies. Nemer and Lindsay (1969) have reported that the incorporation ratio of tryptophan/arginine in nascent protein was substantially less in the s-polysomes than in the rest of the polysomal population. The absence of tryptophan in chromosomal histones (Mirsky and Pollister, 1946; Hnilica, 1967) suggests that the tryptophan-deficient nascent proteins of the s-polysomes may include nascent histones, in accordance with the rationale of Borun *et al.* (1967), bearing on their observations with mammalian tissue culture cells. A similar tryptophan/lysine asymmetry in s-polysomes has been noted recently by Kedes *et al.* (1969), together with the observation through autoradiography that preponderantly nuclear protein is synthesized in the early sea urchin embryo.

In order to assign a specific function to the newly accumulating class of s-polysomes of the early stage embryo, we have attempted to analyze the polysomal protein products

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