Studies on Liver Messenger Ribonucleic Acid*

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Strong evidence has accumulated in recent years in favor of the following scheme of protein biosynthesis. DNA directs the formation of complementary strands of messenger RNA which possess the necessary genetic information to determine the sequential arrangement of amino acids in polypeptide chains (1, 2). The messenger RNA attaches itself to ribosomes to form a multiple ribosomal structure, the polyosome or ergosome (3, 4). The amino acids in an activated state are transported by transfer RNA molecules to the polysomes where the genetic message is decoded during movement of messenger RNA relative to the ribosomes. Each ribosome of a polysome constitutes a condensing site through which the messenger RNA passes with successive exposure of codons and at which a polypeptide chain is growing site through which the messenger RNA passes with successive ribosomes. Each ribosome of a polysome constitutes a condensing site through which the messenger RNA passes with successive exposure of codons and at which a polypeptide chain is growing (3, 5-7). Accumulated evidence suggests that the codons consist of three consecutive bases (triplet code) (8-10).

It has been shown that bacterial messenger RNA has a rapid metabolic turnover and that its rate of synthesis is faster than that of transfer RNA and ribosomal RNA (11). Mammalian reticulocytes possess a stable template system for protein synthesis (12). However, such template stability is not the rule in mammalian cells and messenger RNA of rat liver has been shown to possess a rapid metabolic turnover (10, 13, 14). Our experiments reported in this paper provide additional information on various properties of rat liver polysomal messenger RNA with particular reference to its molecular size and metabolic turnover. These studies have verified the rapid metabolic turnover of polysomal messenger RNA and have established a half-life of approximately 2 hours for this component. Metabolism of ribosomal RNA has also been investigated.

EXPERIMENTAL PROCEDURE

Animals—Male albino rats of the Holtzman strain weighing approximately 300 g, and male mice of the C57Bl/6J strain weighing approximately 25 g were purchased from commercial sources and fed a commercial stock ration ad libitum. All animals were killed by decapitation after ether anesthesia.

14C-Labeled Compounds—Orotic-6-14C acid hydrate with a specific activity of 3.45 mc per mmole, and n-leucine-1-14C with a specific activity of 22.3 mc per mmole were purchased from the New England Nuclear Corporation.

Preparation of Liver Purified Ribosomes—In the pulse-labeled experiments, rats were treated intraperitoneally with injection 100 mc of orotic-6-14C acid hydrate and killed 4 hours later. Mice received an intraperitoneal injection of 15 mc of orotic-6-14C acid hydrate and were killed at the indicated times. Livers were excised immediately after killing and homogenized in Hoagland's medium A (0.005 M Tris, 0.025 M KCl, 0.005 M MgCl₂, and 0.25 M sucrose) with a motor-driven Potter-Elvehjem tissue grinder fitted with a Teflon pestle. All isolation procedures were performed at 0-2°C. Purified ribosomes were obtained by centrifugation of the deoxycholate-treated postmitochondrial fraction through two layers of sucrose (15).

Fractionation of Ribosomes into Polysomes of Varying Aggregate Size—Ribosomes were layered as an inverted gradient (sucrose, 0 to 0.25 M) over 28 ml of a sucrose linear gradient (0.3 to 1.0 M). To obtain these gradients, sucrose was dissolved in Hoagland's salt buffer. After centrifugation for 4 hours at 25,000 r.p.m. and 1°C in the SW 25 Spinco rotor, 1-ml fractions were collected from a needle inserted through the bottom of the centrifuge tube and their absorbances at 254 μM determined. Polysomes of varying aggregate sizes were isolated as pellets by centrifugation of these fractions for 2 hours at 105,000 x g.

Preparation of RNA from Ribosomes—RNA was liberated from ribosomes by treatment with sodium dodecyl sulfate according to the method of Kurland (16) with minor modifications. Of 0.5% SDS, 1 ml was added to a ribosomal pellet derived from approximately 1 g of liver. The suspension was stirred continuously with a Potter-Elvehjem all-glass homogenizer for 3 minutes at 37°C, and then layered over 30 ml of a cold linear sucrose gradient (0.5 to 1.0 M) containing 0.005 M Tris-HCl, pH 7.2. After centrifugation for 40 hours at 25,000 r.p.m. and 1°C in the SW 25 Spinco rotor, 1-ml fractions were collected from a needle inserted through the bottom of the centrifuge tube and their absorbances at 254 μM determined. Sedimentation constants of RNA fractions were determined by calibrating the sucrose linear gradient with the 18 S and 29 S ribosomal RNA (10).

Preparation of RNA from Purified Nuclei—Rat liver nuclei were isolated and purified by the citric acid method (17). Of 1% SDS, 3 ml were added to nuclei isolated from approximately 1 g of liver and the suspension stirred continuously for 30 minutes at 0-2°C in a motor-driven Potter-Elvehjem tissue grinder fitted with a Teflon pestle. An equal volume of water-saturated phenol was then added and stirring continued for another 30 minutes at 0-2°C. The resulting emulsion was centrifuged for 10 minutes at 4000 r.p.m. in a refrigerated centrifuge and the aqueous layer together with the viscous interphase zone extracted.

This purified preparation will be referred to as ribosomes throughout the paper.
for 15 minutes with phenol as described above. The aqueous layer was reextracted with phenol for 10 minutes and, after centrifugation, freed from residual phenol by quick extraction with ether. Addition of ether causes some precipitation of SDS and the aqueous layer appears as a white emulsion. The aqueous phase, clarified after removal of ether by bubbling with nitrogen and subsequent stirring for 3 minutes at 50°, was layered over the cold sucrose linear gradient, centrifuged, and fractionated as described above for rRNA.

Measurement of Radioactivity—After their absorbances were determined, the collected 1-ml fractions of RNA were assayed for their radioactive RNA content by precipitation with HClO₄ according to the method of Stachelin et al. (10) with minor modifications. Of 0.6 N HClO₄, 1 ml was added to each sample after addition of 20 mg of Celite⁴ and 0.5 ml of a bovine albumin solution (4 mg per ml) serving as a coprecipitant. Resulting precipitates were collected by suction on filter paper disks (Whatman No. 540) layered with 0.1 mg of Celite in a stainless steel filtration apparatus⁵ and washed successively with 6 ml of 0.5 N HClO₄, twice with 5 ml of isopropanol-ethyl ether (1:2), and finally with 5 ml of isopropanol-ethyl ether-chloroform (2:2:1). Dried precipitates were transferred to glass-counting vials and 1 ml of hydroxide of Hyamine⁶ solution was added. After 1 hour at room temperature, 9 ml of a 0.05% solution of 2,5-di-phenyloxazole in toluene were added and radioactivity measured in a Packard Tri-Carb scintillation counter.

Cell-free Amino Acid Incorporating System—Ribosomes were suspended in 0.4 ml of Hoagland’s salt buffer and 0.1 ml of each of the following solutions was added (all amounts given per ml): (a) 32 μmoles of MgCl₂, 300 μmoles of Tris-HCl, pH 7.6, and 20 μmoles of β-mercaptoethanol; (b) 100 μmoles of phosphoenolpyruvate (tricyclohexylamine salt purchased from the Sigma Chemical Company) and 0.1 mg of pyruvate kinase (purchased from the Sigma Chemical Company); and (c) 10 μmoles of ATP and 4 μmoles of GTP. The incorporation mixture was completed by addition of 0.1 μmole of N-leucine-1-¹⁴C in Hoagland’s salt buffer, 0.1 ml of dialyzed postmicrosomal supernatant, and Hoagland’s salt buffer to a final volume of 1 ml. The postmicrosomal supernatant was derived from a 25% rat liver homogenate in Hoagland’s medium A after removal of the microsomal fraction (18). This supernatant was dialyzed at 0° for 24 hours against a 50-fold volume of Hoagland’s salt buffer containing 0.005 M β-mercaptoethanol. Optimum incorporation was obtained with this system containing ribosomes derived from 0.6 to 0.8 g of liver and 0.1 ml of dialyzed supernatant. After incubation at 37° for the desired time, incorporation was stopped by addition of 1 ml of 1 M NaOH. The mixture was kept at room temperature for 1 hour to remove radioactive leucine bound to RNA, and the protein was then precipitated with HClO₄, filtered, washed, solubilized with Hyamine, and radioactivity measured in a Packard Tri-Carb scintillation counter (13). Controls utilized in each experiment were kept at 0° and 1 M NaOH was added at zero time. Counting efficiency was approximately 54% for ¹⁴C. Maximum deviation between duplicate samples above 100 c.p.m. was <10% of the mean while that above 1000 c.p.m. was <5% of the mean.

Concentration of Mg⁺⁺ represents a critical factor in the resolution of RNA components, optimum resolution being obtained in the absence of the ion. Presence of Mg⁺⁺ in both the SDS solution and the sucrose gradient (Fig. 1B) resulted in a poor resolution of rRNA as compared with that obtained in the absence of this ion (Fig. 1A). In pulse-labeled experiments, we have observed that most of the counts sedimented to the bottom of the centrifuge tube when Mg⁺⁺ was present in both the SDS solution and the sucrose gradient. This phenomenon has been noted by others (20). The presence of 5 × 10⁻³ M Mg⁺⁺ only in the SDS solution resulted in the disappearance of the radioactive area b- attributed to mRNA (see discussion of data in Fig. 2 below). It has been suggested that mRNA binds to the

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Footnotes:

1. Diatomaceous silica product, Johns-Manville.
2. E-8 B filtration apparatus (Tracerlab, Inc.).

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RESULTS AND DISCUSSION

Extraction and Resolution of RNA—Many factors involved in the techniques of RNA extraction and separation from ribosomes must be controlled in order to ensure maximum resolution of rRNA and, at the same time, avoid degradation of RNA, particularly mRNA. The preparation of ribosomes utilized in our studies constitutes a suitably purified and stable source of RNA. Thus, repeated experiments in our laboratory have shown that preincubation of these ribosomes in Hoagland’s salt buffer at 37° for as long as 10 minutes had no appreciable effect upon their capacity to incorporate N-leucine-1-¹⁴C. Our procedure for extraction of RNA from ribosomes was performed at 0-2° with the exception of one step which entailed exposure of the ribosomes to 37° for 3 minutes in 0.5% aqueous SDS. It would be expected, therefore, that this method affords adequate protection to the RNA associated with ribosomes. We have also established that this procedure achieved quantitative extraction of RNA and that the mass ratio of 29 S to 18 S rRNA ranged between 2.2 and 2.5. These values are in close agreement with those described for Ehrlich ascites tumor cells (19).

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Fig. 1. Effect of Mg⁺⁺ on sedimentation patterns of ribosomal RNA extracted from liver-purified ribosomes. A, ribosomal RNA extracted with 0.5% SDS as described in the text; B, ribosomal RNA extracted from an aliquot of the same ribosomes with 0.5% SDS containing 5 × 10⁻³ M MgCl₂. The same concentration of MgCl₂ was also present in the sucrose linear gradient.
30 S subunit of the ribosome (21) and supportive evidence for this suggestion has been given by binding studies between ribosomes and synthetic polyribonucleotides in presence of Mg++ (22, 23). It seems, therefore, that the presence of Mg++ in the SDS solution used for extraction prevents dissociation of mRNA from ribosomal RNA, particularly the 18 S component (10).

To a lesser degree, resolution also depends upon the quantity of mRNA involved. In our experiments, best resolution was obtained with ribosomes isolated from 0.5 to 1.0 g of liver. Reproducibility of resolution depends upon strict adherence to stated conditions of centrifugation time and composition of the sucrose gradient.

Identification of mRNA—Experiments to be reported in this and succeeding sections were repeated at least three times. Although minor variations existed between experiments, the observed trends were comparable and results of only typical experiments are presented.

Fig. 2 illustrates the sedimentation pattern of pulse-labeled RNA extracted from liver ribosomes. The diagram clearly shows three radioactive peaks, a, b, and c, and an area between b and c with high radioactivity. The radioactive Peaks a and b coincide with the 29 S and the 18 S rRNA, respectively. Peak c which corresponds to 5 S RNA probably consists chiefly of transfer RNA. This is supported by the presence of a single radioactive peak in pulse-labeled RNA extracted from the post-microsomal supernatant by the cold phenol method (10) which coincides with Peak c. These data are in agreement with recent results (10, 20). The radioactive area b–c possesses a high specific activity and is obviously heterogeneous with components possessing sedimentation rates ranging from approximately 4.5 S to 20 S. It will be demonstrated below that this area overlaps with the 18 S rRNA and 5 S RNA components. These characteristics have been assigned to bacterial as well as mammalian mRNA (1, 2, 10).

Further support to the representation of the b–c area as mRNA was given by the following experiments. Preincubation of ribosomes with all the reagents of the amino acid incorporation system in vitro (except nl-leucine-14C) at 37°C for 45 minutes decreased dramatically the content of heavier ribosomal aggregates and produced a parallel increase in amount of 73 S ribosomes (Fig. 3). These preincubated ribosomes retained very little of their original capacity to incorporate nl-leucine-14C (Fig. 4). Ribosomes, 73 S, isolated from the corresponding fraction of preincubated ribosomes by centrifuging for 2 hours at 105,000 × g were unable to incorporate nl-leucine-14C (Fig. 4). The shift from heavy ribosomal aggregates to 73 S ribosomes after preincubation has been described (6) and, in accordance with the tape mechanism of protein biosynthesis (5–7), can be attributed to the continued read-out process of mRNA which results in the release of 73 S monomers held together by the mRNA strand in the polysome. Fig. 5 records the sedimentation pattern of pulse-labeled RNA extracted from 73 S ribosomes isolated from ribosomes preincubated at 37°C for 45 minutes in the amino acid incorporation mixture. Since these ribosomes were isolated from the liver of the rat utilized to provide data of Fig. 2, a direct comparison of the two patterns can be made. It is apparent that the radioactive area between b and c attributed to mRNA (Fig. 2) is not present in RNA isolated from the 73 S ribosomal fraction of preincubated ribosomes (Fig. 5). The fact that the specific activity of Peak b in Fig. 5 is considerably lower than that of the corresponding peak of Fig. 2 provides evidence that the mRNA fraction overlaps with that of 18 S rRNA (see also Table I). The specific activity of Peak c is also lower in Fig. 5 than in Fig. 2. This results from the overlapping of mRNA with this peak as well as from the loss of sRNA during preincubation (10).

The coincidental effects of preincubation upon liver ribosomes, disappearance of radioactive area b–c in the 73 S ribosomal fraction, and disaggregation of ribosomal aggregates with loss of their ability to incorporate amino acids in vitro provide support for the belief that the fraction of ribosomal RNA represented by the b–c area in Fig. 2 is indeed mRNA. This fraction is extremely sensitive to ribonuclease and disappears after treat-
Synthesis of mRNA—In the following experiment, the sedimentation patterns of mRNA species associated with polysomes of varying aggregate size have been determined. Results presented in Fig. 7 showed clearly that the peak of mRNA shifted toward lower sedimentation values as the size of the ribosomal aggregates decreased (10). This result was not unexpected since the 73S ribosomes are bound together in the polysome by the mRNA strand and, obviously, a larger number of 73S monomers in a polysome would require a longer strand of mRNA.

Our data demonstrated that the mRNA fraction consisted of a

**Table I**

Specific activities of rRNA and mRNA associated with rat liver ribosomes after 4-hour labeling with uracil-6-14C nucleotide acid hydrate

<table>
<thead>
<tr>
<th>Sedimentation Rate, Svedberg Units</th>
<th>Specific Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>29S rRNA</td>
<td>3.9 × 10^6</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>5.0 × 10^5</td>
</tr>
<tr>
<td>mRNA</td>
<td>3.2 × 10^5</td>
</tr>
</tbody>
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* Specific activities are expressed as counts per minute per mg of RNA.
† Not calculated.

Department of ribosomes with only 1.2 × 10^-8 g of this enzyme per ml incubation mixture (10).

Sedimentation patterns of pulse-labeled RNA from liver ribosomes and liver nuclei are compared in Fig. 6. A nuclear RNA fraction which has been found to possess a high specific activity and obviously heterogeneous sedimentation behavior sedimented in the same area as that assigned to polyosomal mRNA. It seems likely that this fraction consists of mRNA molecules synthesized in nuclei. A similar nuclear RNA fraction can stimulate amino acid incorporation in vitro by ribosomes (24).

**Figure 4**

Incorporation in vitro of DL-leucine-1-14C by purified ribosomes, ribosomes preincubated at 37°C for 45 minutes (see Fig. 3), and 73S ribosomes isolated from the preincubated ribosomes. Ribosomes were derived from 0.6 g of liver and incorporation in vitro of DL-leucine-1-14C determined by the method described in “Experimental Procedure.”

**Figure 5**

Sedimentation pattern of pulse-labeled RNA extracted from 73S ribosomes. Liver ribosomes were obtained from the same rat utilized to obtain data of Fig. 2 and preincubated as described in Fig. 3. Ribosomes, 73S, were isolated from the corresponding fraction of preincubated ribosomes (Fig. 3) by centrifuging for 2 hours at 105,000 × g. Extraction and fractionation of RNA have been described (Fig. 2). Absorbance measurements are represented by the solid line and radioactivity measurements by the dotted line.

**Figure 6**

Sedimentation pattern of pulse-labeled RNA from liver purified ribosomes and liver nuclei. Ribosomal RNA patterns were obtained as described in Fig. 2. Absorbance is represented by the solid line and radioactivity by the broken line. Nuclei were obtained from liver of a rat administered 100 μg of uracil-6-14C acid hydrate intraperitoneally by injection and killed 4 hours later. Nuclear RNA was fractionated and radioactivity measurements (represented by the dotted line) made as described in “Experimental Procedure.”
FIG. 7. Sedimentation patterns of pulse-labeled RNA extracted from ribosomal aggregates of varying size. Liver ribosomes were prepared from a rat given 100 μg of orotic-6-14C acid hydrate by injection after a 4-hour labeling period and fractionated into polysomes of varying aggregate size as described in "Experimental Procedure." The heaviest aggregates are present in the F1 fraction and the aggregate size decreases as the fraction number increases. The F10 fraction, containing aggregates of the smallest size, consists of a mixture of 113 S and 73 S ribosomes. RNA was extracted from these different fractions, separated on a linear sucrose gradient, and radioactivity was determined as described previously. Range of sedimentation of components of mRNA present in each polysome fraction is indicated by the shaded bar area.

It has been determined that mRNA constitutes approximately 1.3% of total ribosomal RNA (10). Specific activities of rRNA and mRNA have been calculated (Table I) by employing this relationship together with the observed radioactivity data and the quantity of rRNA computed from the absorbance data.
Fig. 8. Sedimentation patterns of pulse-labeled RNA from mouse liver ribosomes and nuclei. Eight C3H/BL male mice were each given 15 μg of orotic-6-14C acid hydrate by injection and one animal was killed at each of the indicated times. Methods for the preparation and fractionation of ribosomal and nuclear RNA have been described previously (Figs. 2 and 6). Absorbance and radioactivity measurements of ribosomal RNA are indicated by solid and broken lines, respectively. Radioactivity measurements of nuclear RNA fractions taken from 15 minutes to 2 hours post-injection of labeled orotic acid are indicated by the dotted lines in the top panels.

Turnover of mRNA—Estimations of the metabolic turnover time of mouse liver mRNA were made from experiments in which specific activities were determined at varying times after the injection of orotic-6-14C acid hydrate. Results of a typical experiment in which the labeling time was varied from 15 minutes to 24 hours is shown in Fig. 8. Inspection of the various sedimentation patterns shows that labeled mRNA appeared rapidly in the polysome population and attained a maximum level of radioactivity in approximately 4 hours which remained constant until termination of the experiment at 24 hours. This is clearly evident in Fig. 9 which records the specific activities of mRNA computed from the data of Fig. 8. According to the tape mechanism of protein synthesis (5-7, 20, 27) the read-out of genetic information is accomplished through a continuous successive attachment of single ribosomes to one end of the mRNA strand in correspondence to their release at the other end with the completed polypeptide chain. This balance of attachment and detachment of ribosomes maintains the polysomal population in a steady state. Concomitantly, disaggregation of polysomes into individual ribosomes resulting from mRNA turnover is balanced by formation of new polysomes with newly synthesized mRNA strands. Thus, a steady state of mRNA is established through equivalence of mRNA breakdown and release into the cytoplasm. According to our data, the specific activity of mouse liver mRNA reached a maximum value approximately 4 hours after the injection of orotic-6-14C acid hydrate. On the basis of the existence of a steady state of mRNA, we can interpret these results to indicate that the mRNA of the entire polysome population is renewed approximately every 4 hours. An average half-life of approximately 2 hours for the spectrum of mouse liver mRNA can, therefore, be derived.

On the other hand, sedimentation patterns presented in Fig. 8 show clearly that the synthesis of rRNA is a slower process than that of mRNA and that the level of radioactivity of rRNA increases continuously until termination of the experiment at 24 hours. Specific activities of 18 S and 29 S rRNA, computed from data of Fig. 8, are presented in Fig. 10. The specific activities of the 18 S component are always somewhat greater than those of the 29 S component since the sedimentation pattern of the 18 S component overlaps with that of mRNA. However, as we have indicated previously in this paper, the true values for the 18 S component are approximately equal to those of the 29 S component.

The metabolic turnover of mRNA is far more rapid than that of rRNA with the differences in specific activity diminishing with increases in pulse-labeling times. Thus, the ratio of specific activities of mRNA to rRNA decreases from 200 at a
The sedimentation pattern of pulse-labeled nuclear RNA observed between 15 minutes and 2 hours subsequent to the injection of orotic-6-\(^{14}\)C acid hydrate resembled that of pulse-labeled mRNA (Fig. 8).

1. Ribosomes have been prepared from rat and mouse liver in a purified and stable state which permitted their utilization as a suitable source for extraction of associated RNA. Optimum resolution of RNA components on a linear sucrose gradient was achieved in the absence of Mg\(^{++}\).

2. Sedimentation patterns of RNA isolated from ribosomes of rats administered orotic-6-\(^{14}\)C acid hydrate by injection revealed a radioactive area which possessed high specific activity and was obviously heterogeneous, with components possessing sedimentation rates ranging from approximately 4.5 S to 20 S. Support for the belief that this fraction represented messenger RNA was derived from experiments utilizing ribosomes preincubated with the components of an amino acid incorporating system \textit{in vitro}. Under these conditions, the ribosomal aggregates were broken down with concomitant loss of their ability to incorporate amino acids \textit{in vitro}. Furthermore, the 73 S ribosomes isolated from these preincubated ribosomes contained no measurable amounts of the radioactive fraction attributed to messenger RNA.

3. Sedimentation patterns of mRNA species associated with polysomes of varying aggregate size have been determined. The peak of mRNA shifted toward lower sedimentation values as the size of the ribosomal aggregates decreased. The mRNA component of liver polysomes consisted of a spectrum of molecules with sedimentation constants ranging from approximately 4.5 S to 20 S corresponding to a molecular weight range of 30,000 to 800,000. With the existence of a triplet code, this spectrum of liver mRNA molecules can code for proteins containing 30 to 800 amino acids.

4. In pulse-labeled experiments, the 29 S and 18 S components possessed the same specific activities. Since the specific activities of these components were the same in ribosomal aggregates of varying size, we have concluded that newly synthesized ribosomes are distributed statistically among the polysomal population.

5. Estimations were made of the metabolic turnover of mouse liver mRNA in pulse-labeling experiments with orotic-6-\(^{14}\)C acid hydrate. The specific activity of mRNA reached a maximum value approximately 4 hours after the injection of the labeled orotic acid. These results were interpreted, on the assumption that mRNA is in a steady state, to indicate that the mRNA of the entire polysomal population is renewed approximately every 4 hours. An average half-life of approximately 2 hours for mouse liver mRNA was, accordingly, derived. The
metabolic turnover of mRNA was far more rapid than that of ribosomal RNA. The ratio of specific activities of mRNA to ribosomal RNA decreased from 200 at a pulse-labeling time of 1 hour to 30 at a pulse-labeling time of 24 hours.

6. Sedimentation patterns of pulse-labeled RNA from liver ribosomes and nuclei have been compared. We have observed a nuclear RNA fraction which possessed a high specific activity and an obviously heterogeneous sedimentation behavior. This fraction sedimented in the same area as that assigned to poly-somal mRNA.

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REFERENCES