*AN ESTIMATION OF GENETIC MESSAGES IN THE UNFERTILIZED ECHINOID EGG**

By D. W. Slater† and S. Spiegelman

DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF ILLINOIS, URBANA

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The metabolic inactivity of the mature echinoid egg is abruptly terminated at fertilization.[1, 2] The vigorous onset of protein synthesis poses the problem of whether the inhibition released by fertilization is at the level of the transcription or the translation of genetic information; a rephrasing in modern terminology of 50 years of speculation regarding the existence of cytoplasmic information in the unfertilized egg.

Recent indirect attempts to resolve this issue have shown that the initiation of protein synthesis is immune to either the presence of actinomycin[3, 4] or the absence of nuclear DNA,[5] implying that tandem transcription is unnecessary. Such observations are readily interpretable in terms of preformed genetic messages necessary to the beginning of embryogenesis.

It is evident that a more direct demonstration would be desirable. A pioneering preliminary attempt in this direction was made by Maggio *et al.*,[6] who reported a stimulation of amino acid incorporation by RNA from unfertilized eggs in a heterologous cell-free system. Unfortunately, the slight stimulation of amino acid incorporation obtained by these investigators, barely onefold above background, necessitated massive amounts of RNA (up to 1.2 mg). This low level of response could have resulted from bacterial contamination[7] and/or the template activity associated with partially degraded ribosomal RNA.[8]

In view of the importance of the issues involved, it seemed desirable to re-examine this question with the more rigorous methods now available. A decisive, positive outcome requires an assay of the *amount* of translatable RNA from the unfertilized egg, using a calibrated system standardized against an RNA of known message content. Further, some guarantee must be provided that the RNA removed from the unfertilized egg has not suffered degradation, a process which could possibly generate translatable artifacts. It is the purpose of the present paper to present the pertinent experiments. The data indicate that 4–5 per cent of the bulk RNA derived from unfertilized eggs possesses the ability to serve as templates for protein synthesis.

Materials and Methods.—(1) *Preparation of unfertilized eggs:* The sea urchin employed was *Lytechinus pictus*. Partial shedding was induced by 0.55 *M* KCl and the ova were extensively washed by both sedimentation and centrifugation in artificial sea water.[9] The eggs were examined for the presence of cytolyzed material, immature oöcytes, and such other contamination as is common to echinoid material. Aliquots of each batch of eggs were fertilized and those which did not yield better than 95% normal development were discarded. Following the final centrifugation, the eggs were frozen in a dry-ice methanol bath and stored at −70°C.

(2) *Preparation of unfertilized egg RNA:* Unfertilized egg RNA (UF-RNA) was extracted as detailed elsewhere,[10] with the exceptions noted below. The final concentration of sodium dodecyl sulfate was 0.5%, rather than 1%. All of the UF-RNA employed was prepared via 4°C phenol extraction rather than by the 60°C phenol procedure. Although hot phenol preparations allegedly yield products which exhibit enhanced template potential, this technique leads to some degradation and aggregation.[10] It has also been reported that thermal distortion of ribosomal and

transfer RNA secondary structure can render these species of cellular RNA capable of functioning as templates for protein synthesis. The procedure utilized yielded a product which contained no UV-absorbing RNase-resistant material as judged by sucrose sedimentation analysis. It should also be noted that residual DNase I was present in the S30 extracts (see below). The possibility of DNA-directed protein synthesis[11] was eliminated by subjecting the RNA to DNase I (electrophoretically pure, Worthington Biochemical Corp., Freehold, N. J.) as previously described.[10] All UF-RNA preparations utilized in this study exhibited 260/230 mμ and 260/280 mμ extinction ratios of greater than 2.0.

(3) *Preparation of MS-2 and chloramphenicol RNA:* MS-2 RNA was prepared as previously described.[12] Chloramphenicol RNA (CM-RNA) was prepared from *E. coli* (K10). The cells were grown to an optical density at 660 mμ of approximately 0.3 and subsequently incubated with 50 μg per ml of chloramphenicol for 30 min. Following exposure to chloramphenicol the cells were harvested and the RNA was extracted as detailed elsewhere.[13]

(4) *Sucrose density gradient centrifugation:* Sucrose density gradient centrifugation analysis was carried out as previously described.[10] Centrifugation was for 10 hr at 24,000 rpm in the SWB 25.1 rotor of the Spinco model L preparative ultracentrifuge. The temperature during centrifugation was approximately 2–3°C.

(5) *Preparation of S30 fractions: E. coli* S30 extracts were prepared from the mutant Hfr strain Q13, a derivative of A19.[14] Q13 displays less than 0.2% of the RNase I activity characteristic of the wild type and shows no polynucleotide phosphorylase activity. The cells were grown under aeration in a culture medium previously described.[15] Growth was terminated in early log phase by the addition of crushed ice. The cells were harvested and washed twice in the reaction mixture solution (RMS) which contained 0.01 *M* tris buffer pH 7.8; 0.012 *M* magnesium acetate; 0.012 *M* KCl; 0.05 *M* ammonium acetate; and 0.006 *M* β-mercaptoethanol. The resulting pellet was stored at −70°C.

Cell-free extracts were prepared as described below. Frozen cells were placed in the chamber of a Hughes press[16] which had been precooled at −20°C. Cells were ruptured by applying approximately 10,000 psi and subsequently resuspended in RMS at 4°C. Following disruption, DNase I was added to a final concentration of 15 μg per ml. Intact cells and debris were pelleted by centrifugation at 15,000 × *g* for 10 min. The supernatant was removed and centrifuged for 30 min at 30,000 × *g*. The resulting supernatant was aspirated off and henceforth will be referred to as the S30 fraction. All S30 fractions were preincubated at 35°C for 45 min with the following additions per 10 ml of extract: 0.75 ml of a 0.12 *M* phosphoenolpyruvate kinase; 0.75 ml of a solution containing 0.03 *M* ATP, and 0.006 *M* GTP, sodium salts; and 0.4 ml of a solution containing 4×10^{-5} *M* of each of the commonly occurring L-amino acids. Subsequent to this preincubation, the mixture was dialyzed against RMS (100:1) for 10 hr at 4°C with four changes of buffer. The S30 extracts were then centrifuged at 20,000 × *g* for 15 min and the supernatant was divided into small samples which were stored at −70°C.

It should also be noted that S30 preparations obtained from cells disrupted via sonication yielded extracts which were significantly more active than those prepared from cells broken in the Hughes press. However, this procedure also resulted in a 50% increment in residual nuclease activity.[17] As such latent RNase II might be instrumental in either the masking of existing messenger RNA via degradation and/or theoretically permitting the translation of otherwise inactive templates, all of the results reported herein were obtained with S30 Hughes press preparations.

(6) *Amino acid incorporation assay:* The reaction mixture contained the following components: 0.3 μM of ATP; 0.06 μM of GTP; 1.2 μM of PEP; 10 μg of PK; 0.01 ml of a solution containing 4×10^{-5} *M* of each of the commonly occurring amino acids except serine; 0.03 ml of the S30 extract; and 0.03 ml of a solution consisting of 42.5 μc per 1.05 μM C^{14}-serine (New England Nuclear Corp., Boston, Mass.). The final volume of the reaction mixture was adjusted to 0.2 ml by addition of the appropriate amount of RMS containing from 0 to 100 μg of the RNA to be assayed. Incubation was carried out at 35°C for either 40 min, or, in the case of kinetic assays, for the time indicated. The reaction was terminated with cold 5% TCA containing 1% casamino acids. Samples were heated for 20 min at 90°C and subsequently chilled in ice. Each precipitate was collected on a filter membrane (B6-27 mm of Schleicher and Schuell, Keene, N. H.) and extensively washed with cold 5% TCA. The membranes were thoroughly dried and subsequently counted in a liquid scintillation spectrometer (Packard Instrument Co., Des Plaines, Ill.) with an

efficiency of 30%. Under the conditions employed, 1000 cpm represented an incorporation of 38 $\mu\mu$M of C^{14}-serine.

Results.—Standardization of the system for the assay of translatable RNA: Evidence is first presented on the quantitative reliability of our procedure for estimating template activity. Two sources of translatable RNA were employed in calibrating the system. One was bulk RNA (CM-RNA) prepared from chloramphenicol-treated *E. coli*. Such preparations possess three to four times more template potential than those extracted under otherwise similar conditions from normal log-phase cells.[18] This RNA provides a suitable source of mixed natural messages which can be used to characterize the protein-synthesizing abilities of the cell-free system. The other RNA used is derived from purified RNA bacteriophage (MS-2) which can be taken as a preparation of pure message.

Figure 1 shows the kinetics of the incorporation in the presence of 50 μg CM-RNA. It will be seen that the kinetics proceed in a linear fashion for approximately 20 min, reaching a plateau after about 40 min. It is also evident that the system is strictly dependent on the addition of added RNA. Similar responses were obtained with C^{14}-histidine, C^{14}-valine, and H^3- or C^{14}-leucine.

Figure 2 shows the strict proportionality between the observed incorporation in the standard assay (*Methods*) and the amount of input RNA from 0 to 80 μg. Comparison of the activity of CM-RNA (Fig. 2) with that obtained with MS-2 RNA (Fig. 5) reveals that the average relative template activity of the CM-RNA used in these experiments is approximately 9 per cent of that of MS-2 RNA.

The template potential of RNA from the unfertilized egg: The physical integrity of the RNA prepared from unfertilized eggs by the 4°C phenol extraction procedure (*Methods*) was established by sucrose density gradient centrifugation (Fig. 3). Approximately 94 per cent of the RNA is of a non-4*S* nature. The sedimentation

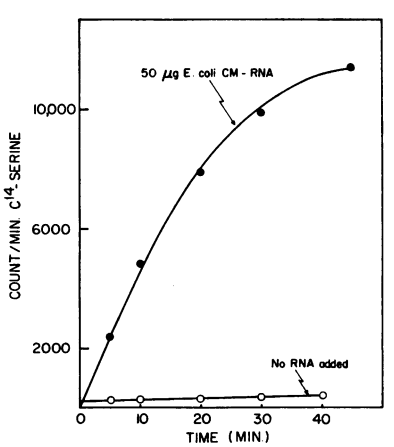

FIG. 1.—Kinetics of *E. coli* CM-RNA-directed C^{14}-serine incorporation. A concentration of RNA was employed (50 μg per tube) which was within the established range of linearity. The reaction conditions were as given in *Methods*. Incorporation was terminated at the time indicated and proceeded in a linear fashion for approximately 20 min.

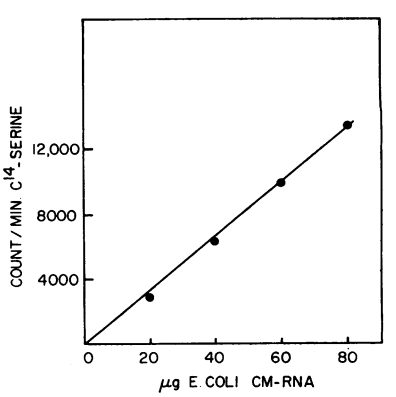

FIG. 2.—Effect of *E. coli* CM-RNA concentration on C^{14}-serine incorporation. Reaction conditions were as given in *Methods*. Incorporation was proportional to the quantity of input CM-RNA through 80 μg. The addition of 80 μg of CM-RNA resulted in the incorporation of 13,488 cpm of C^{14}-serine (513 $\mu\mu$M) above that incorporated in the absence of added RNA (*background*). A background value of 461 cpm has been subtracted from all of the points plotted in this figure.

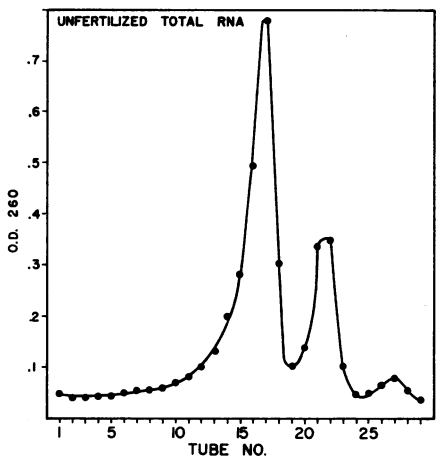

FIG. 3.—Sedimentation analysis of unfertilized egg total RNA. Bulk UF-RNA was prepared via 4°C phenol extraction. The RNA was layered on a 5–20% sucrose gradient and centrifuged at 24,000 rpm for 10 hr, as detailed elsewhere.[10] The major components possessed sedimentation coefficients of 28*S*, 17–18*S*, and 4*S*, respectively. Of the 4.3 optical density units employed, 96% exhibited sedimentation values of greater than 8*S*.

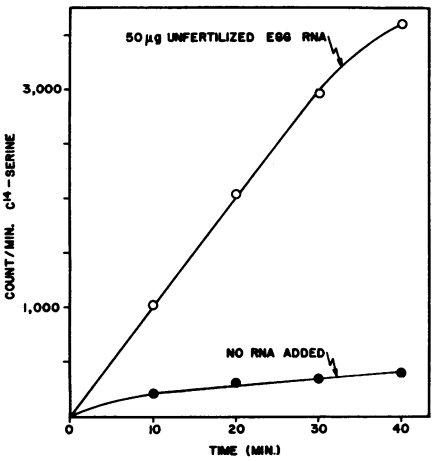

FIG. 4.—Kinetics of unfertilized egg RNA-directed C^{14}-serine incorporation. A concentration of RNA was employed (50 µg per tube) which was within the established range of linearity (Fig. 1). Reaction conditions were as given in *Methods*. Each reaction was terminated at the time indicated. No lag in the initiation of synthesis was observed. Incorporation was linear through 30 min.

profiles are dissimilar to those of either *E. coli* or *B. megaterium*[10] in that the heaviest major component exhibits a sedimentation value of 28*S*, in contrast to the bacterial RNA's 23*S*. Thus, in addition to the obvious physical integrity of the RNA, the discreet banding shown in Figure 3 and the aforementioned sedimentation value provisionally preclude the presence of substantial bacterial contamination.

The kinetics of UF-RNA-directed amino acid incorporation are presented in Figure 4. No lag in the initiation of synthesis is observed, the only anomalous aspect of the kinetic pattern being the somewhat extended period during linear incorporation. In the case of CM-RNA, linearity was generally lost subsequent to 20 min of incubation, whereas the UF-RNA maintains a linear rate through 30 min. The response of the system to increasing amounts of UF-RNA is shown in Figure 6, from which it is evident that UF-RNA is capable of linearly stimulating amino acid incorporation through 100 µg per reaction mixture, resulting in a 15-fold increment above background.

The estimation of the amount of template activity in the RNA from the unfertilized egg: A quantitative index of the ability of RNA derived from unfertilized eggs to stimulate amino acid incorporation was obtained by direct comparison of its protein-synthesizing potential with that of MS-2 RNA (Fig. 5). The specific activity of the UF-RNA (defined as counts per minute of C^{14}-serine incorporated per microgram of input RNA) was 86, within the range of linear response. Concomitantly, MS-2 RNA exhibited a specific activity of 1783. Thus, since MS-2 RNA may be regarded as 100 per cent translatable message, the bulk RNA extracted from the unfertilized eggs possesses a relative template activity of approximately 4–5 per cent.

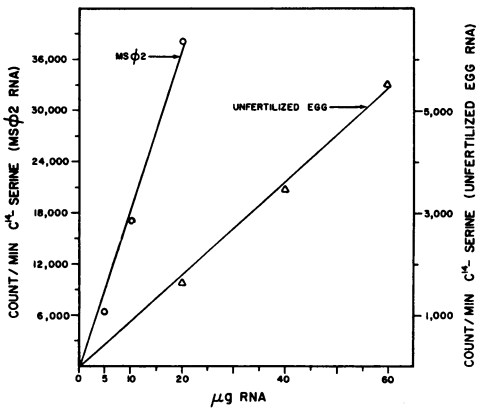

FIG. 5.—Comparative stimulatory capacities of unfertilized egg RNA and MS-2 RNA. UF-RNA and MS-2 RNA were assayed concomitantly. Reaction conditions were as given in *Methods*. The addition of 20 μg of MS-2 RNA resulted in the incorporation of 38,619 cpm of C^{14}-serine (1,163 μμM), whereas the addition of 60 μg of UF-RNA stimulated the incorporation of 5,579 cpm of C^{14}-serine (211 μμM) above that incorporated in the absence of added RNA (*background*). A background value of 608 cpm has been subtracted from all of the points plotted in this figure. The average specific activities of MS-2 RNA and UF-RNA were 1,783 and 86, respectively.

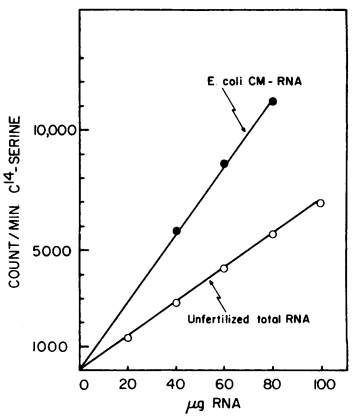

FIG. 6.—Comparative stimulatory capacity of unfertilized egg RNA and *E. coli* CM-RNA. UF-RNA and *E. coli* CM-RNA were assayed concomitantly. Reaction conditions were as given in *Methods*. The addition of 80 μg of CM-RNA resulted in the incorporation of 12,192 cpm of C^{14}-serine (463 μμM), whereas 100 μg of UF-RNA stimulated the incorporation of 6,925 cpm of C^{14}-serine (263 μμM) above that incorporated in the absence of added RNA (*background*). A background value of 487 cpm has been substracted from all of the points plotted in this figure. The average specific activities of the CM-RNA and UF-RNA were 146 and 70, respectively.

As previously mentioned, the CM-RNA employed in our experiments exhibits roughly 9 per cent of the template activity characteristic of MS-2 RNA. This permits an evaluation of the reproducibility of the comparative template activities. Thus, on the basis of the above information, it may be predicted that the UF-RNA should exhibit 50 per cent of the amino acid incorporation activity displayed by CM-RNA. To test this prediction, comparable quantities of CM-RNA and UF-RNA were assayed in parallel. The results are shown in Figure 6. The specific activities of the UF-RNA and CM-RNA were 70 and 146, respectively, thereby being in rather good agreement with the projected value of approximately 50 per cent for the UF-RNA.

The absolute significance of these numbers must be conditioned by a consideration of the translational efficiency of the three types of RNA employed. *E. coli* is a normal host of MS-2, and is also the source from which the CM-RNA was prepared. Therefore, both of these RNA's were functioning in a homologous cell-free extract. Conversely, RNA derived from unfertilized sea urchin eggs was required to stimulate amino acid incorporation in a distinctly heterologous medium. This situation suggests that the observed 4–5 per cent relative template activity displayed by UF-RNA may prove to be a minimal estimate of its actual potential.

Discussion.—The sedimentation profiles of the RNA employed in this paper unequivocally established their physical integrity. The cell-free system utilized (which exhibited a low nuclease content) was monitored and characterized by RNA possessing known message potential. Protein synthesis was time-dependent and

proportional to the amount of RNA added. Under these defined conditions, 100 μg of unfertilized egg RNA stimulated a 15-fold increment in amino acid incorporation. The relative template potential of these RNA preparations proved to be approximately 5 per cent of that displayed by MS-2 RNA, and approximately 50 per cent of that exhibited by CM-RNA.

It is obvious that the numerical relations exhibited completely exclude ascribing the stimulatory response of UF-RNA to bacterial contamination of the unfertilized eggs. Hypothetically, even if the entire biomass of the material used to prepare UF-RNA were *E. coli*, only 50 per cent of the observed response would have been obtained. Thus, bacterial contamination could only serve to diminish the observed message activity. Finally, the *S*-values of the RNA used (Fig. 3) show no detectable evidence of contamination with the 23*S* ribosomal RNA characteristic of bacteria.

The mature echinoid egg does not synthesize RNA during the protracted period of metabolic dormancy preceding fertilization. Clearly, any estimates of the longevity of some metazoan messenger RNA's must therefore be redefined in terms of weeks rather than minutes or hours. It is also apparent that the massive increment in amino acid incorporation which follows fertilization does not mandatorily require transcription. If this transition in translational potential is prefaced by a transcriptive prerequisite of a similar magnitude, then it is reasonable to assume that it might be reflected in a concomitant increase in the comparative template capabilities of ensuing embryonic stages. Evidence will be forthcoming, however, that the pattern of messenger RNA potential during early embryogenesis is not characterized by any gross alteration in the titer of template activity.[19]

Summary.—Bulk RNA prepared from unfertilized echinoid eggs possesses the ability to stimulate protein synthesis in a defined cell-free amino acid incorporating system. Unfertilized egg RNA exhibits approximately 4–5 per cent of the template activity displayed by MS-2 RNA and approximately 50 per cent of that of chloramphenicol RNA. The proportion of translatable RNA found in unfertilized eggs is therefore approximately twofold that found in normal *E. coli* cells growing in log phase. It is concluded that the increment in protein synthesis which follows fertilization is completely explainable in terms of pre-existent messages, and thus does not mandatorily require tandem transcription.

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† Mr. Slater is a predoctoral trainee in Cell Biology USPHS 5-Tl-GM-941-02 MBS.

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