Limited Complexity of the RNA in Micromeres of Sixteen-Cell Sea Urchin Embryos

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Received April 2, 1980; accepted April 11, 1980

The sequence complexity of sea urchin embryo micromere RNA is about 75% of that of total 16-cell embryo cytoplasmic RNA, as reported earlier by Rodgers and Gross [Rodgers, W. H., and Gross, P. R. (1978) Cell 14, 279-288]. In contrast to the rest of the embryo, there are few, if any, complex maternal RNA species in the micromere cytoplasm which are not represented in the polysomes. The micromeres do not contain detectable quantities of high-complexity nuclear RNA, though such RNA exists in other cells of the fourth-cleavage embryo.

INTRODUCTION

At the eight-cell stage of early cleavage, sea urchin embryos consist of four “animal” blastomeres and four “vegetal” blastomeres, all of which are similar in size and form. During fourth cleavage, the animal blastomeres again divide equally, giving rise to eight mesomeres, but the four vegetal blastomeres cleave unequally into four large macromeres and four very small micromeres situated at the vegetal pole of the embryo. All of the blastomeres, including the micromeres, undergo several further rounds of division as the embryo assumes the hollow blastula form. Descendants of the micromeres, the primary mesenchyme cells, then migrate into the blastocoel. Groups of these cells secrete two calcareous granules, upon which the first spicules of the larval skeleton are later constructed (Hörstadius, 1937, 1973). Micromeres that are isolated and maintained in culture appear to follow their normal developmental fate, including the secretion of skeletal spicule elements (Okazaki, 1975). Micromeres thus give rise to a discrete cell lineage, the differentiated character of which is well defined in morphological terms.

The origin of the functional distinction between the micromere cell lineage and the remainder of the embryo is a matter of speculation. Among the possibilities often considered are that the 16-cell embryo contains an unequal distribution of maternal mRNA sequences or of newly synthesized embryo mRNAs. However, Senger and Gross (1978) reported that protein synthesis patterns in the three blastomere cell types are qualitatively similar. Even with the increased resolution made possible by two-dimensional gel electrophoresis, Tuftaro and Brandhorst (1979) detected no clear qualitative differences among the three cell types in the synthesis of more than 1000 different proteins. Thus it would appear that micromeres contain more or less the same set of abundant and moderately abundant mRNAs as does the rest of the 16-cell embryo. On the other hand, Rodgers and Gross (1978) found that the rare single-copy sequence transcripts of the 16-cell embryo are not homogeneously distributed. Their experiments indicated that micromere RNA includes only 65-80% of...
the complex set of low-abundance maternal RNA sequences found in the remainder of the embryo.

In this report we present further measurements of the complexity of micromere total RNA and, in addition, of micromere polysomal RNA. The results imply that, in molecular terms, micromeres differ from the remainder of the embryo in several ways that may be relevant to their separation as a unique cell lineage.

MATERIALS AND METHODS

Sea urchin embryos. Strongylocentrotus purpuratus females were spawned and the eggs washed, collected, and fertilized by standard methods (Smith et al., 1974). Fertilization was at a concentration of about 2–5 × 10⁵ eggs/ml Pen-Strep MPFSW (30 units/ml penicillin, 50 μg/ml streptomycin in Millipore-filtered seawater). The fertilized eggs were demembranated by modifications of standard procedures (Hynes and Gross, 1970). At 90 sec or when 85–90% fertilization was achieved, whichever came first, an equal volume of freshly prepared 0.08% papain, 0.40% glutathione (pH 7.8) was added. The eggs were kept in suspension by gentle swirling, and fertilization membranes began to disappear within 90–120 sec. After 7–9 min, the eggs were diluted to 1–3 × 10⁴/ml and grown at 14–15°C with stirring. Demembranization by this method did not affect the normal development of the embryos to the pluteus stage.

In some experiments RNA was isolated from whole 16-cell embryos. These embryos were demembranated, grown, and dissociated as described for isolation of micromeres. Thirty-two- to 64-cell embryos were cultured by standard procedures (Smith et al., 1974) and harvested at 7.5 hr, when approximately 50% of the embryos had undergone the sixth division and contained 64 cells, while the remainder were still at the fifth-cleavage stage.

Isolation of micromeres. Micromeres were isolated by a procedure similar to that employed by Rodgers and Gross (1978). About 5 hr after fertilization, when the embryos had just completed their fourth division, they were allowed to settle and the seawater was aspirated off. The growing chamber was placed in an ice bath to retard further cell division and “run off” of ribosomes from polysomes. Eight hundred to 1200 ml of ice-cold CMFSW (calcium–magnesium-free seawater) was added to the embryos to dissolve the hyalin layer and dissociate the embryos. Two or three washes of CMFSW were generally sufficient to cause dissociation. Occasionally, blastomeres continued to adhere after several washes with CMFSW, and in this case the solution was brought to 0.5–2.5 mM EDTA or to 1 M glycine (pH 8). Either treatment results in complete dissociation of the blastomeres.

Dissociated blastomeres were adjusted to a concentration of about 3 × 10⁵ embryos/ml of 1% Ficoll 400,000 (Sigma) in CMFSW. Three to 3.5 ml of cell suspension was layered onto 5–15% Ficoll gradients in CMFSW in 30-ml Corex centrifuge tubes. The gradients were centrifuged for 1 min at 200–250g in a Sorvall HB4 swinging-bucket rotor. A discrete band of micromeres formed about 25 mm from the top of the gradient. Micromeres from 8–10 gradients were pooled, concentrated, and, if necessary, sedimented into a second gradient. The micromere band was harvested and a sample containing several hundred blastomeres was counted to determine the purity of the micromere fraction. If less than 99% of the cells were micromeres, the preparation was repurified or discarded.

RNA preparations. Total RNA was extracted from mature eggs, dissociated 16-cell embryos, isolated micromeres, and 32-, 64-cell embryos, according to the procedure of Hough-Evans et al. (1977) for total egg RNA. Briefly, eggs or embryos were homogenized in 7 M urea in low-salt buffer, and the homogenate was extracted with phenol and chloroform:isoamyl alcohol. Nucleic acids were precipitated and the RNA was further purified by DNase and
proteinase K digestion and chromatography on Sephadex G-100. Cytoplasmic RNA was prepared from 16-cell embryos by a similar procedure, after cell lysis and pelleting of nuclei, as previously described (Hough-Evans et al., 1977). Sixteen-cell embryo polysomes were isolated according to Galau et al. (1976). Micromere polysomes were obtained as follows: Isokinetic 5-20% sucrose gradients in 500 mM KCl, 10 mM MgCl₂, 10 mM Pipes [piperazine-N,N'-bis(2-ethanesulfonic acid)] (pH 6.5), 50 µg/ml polyvinyl sulfate, were prepared in 5-ml polycarbonate tubes containing a 0.6-ml cushion of 40% sucrose in the same buffer. Seventy-five to 150 µl of the postmitochondrial supernatant fraction from the isolated micromeres was layered onto the gradients and centrifuged at 50,000 rpm in a Beckman SW 50.1 rotor for 40 min at 4°C. The polysomes were sedimented to the top of the 40% sucrose cushion. Gradients were pumped through an Isco flow cell and monitored for optical absorbance at 254 nm. The <80 S material was discarded and the polysome fraction was diluted three- to fourfold. The polysomes were dissociated by the addition of 0.5 M EDTA to a final concentration of 75-100 mM and incubation for 15 min on ice. The suspension was then layered onto a second sucrose gradient. RNA was extracted from material sedimenting at <80 S as described previously (Galau et al., 1976).

**Single-copy DNA and “egg DNA” tracers.** Total single-copy DNA was prepared as described by Galau et al. (1976). The DNA was labeled in vitro with [³H] by the “gap translation” method with Escherichia coli DNA polymerase I (Boehringer Mannheim) (Galau et al., 1976; Hough-Evans et al., 1977). The single-copy tracers used had specific activities of 0.6-1 × 10⁷ cpm/µg and a weight-average fragment size of 200-250 nucleotides.

Egg DNA consists of that fraction of single-copy DNA sequences which form duplexes with egg RNA. The egg DNA tracers were prepared as previously described. An excess of total egg RNA was reacted with single-copy [³H]DNA (Hough-Evans et al., 1977, 1979) to equivalent RNA C₀t values greater than 50,000. The hybridized [³H]DNA was harvested and further enriched for sequences homologous to egg RNA by a second reaction with egg RNA, followed by isolation of the hybridizing sequences. Four different egg DNA preparations were used in this study.

**Hybridization conditions.** In the reactions described, the RNAs were in 10³- to 10⁴-fold mass excess with respect to [³H]DNA tracers. Hybridization reactions were carried out in 0.41 M phosphate buffer (pH 6.8), 1.5 mM EDTA, 0.05% sodium dodecyl sulfate at 60°C. All C₀t values presented are equivalent C₀t's, i.e., they have been corrected for acceleration in hybridization rate due to Na⁺ concentration greater than 0.18 M (Britten et al., 1974). DNA-DNA and DNA-RNA duplexes were assayed by hydroxyapatite chromatography, essentially according to Galau et al. (1974). After hybridization, samples were diluted in 1.0 ml of 0.05 M phosphate buffer and divided into two aliquots. One-half was brought to 0.12 M phosphate buffer and assayed for total duplex. The other half was incubated with 10 µg/ml RNase A in 0.05 M phosphate buffer for 1 hr at 37°C. It was then brought to 0.12 M phosphate buffer and assayed for DNA-DNA duplex. The difference in the amount of bound [³H]DNA in total duplex and DNA-DNA duplex measures the extent of DNA-RNA hybridization. Values were corrected for reactivity of the [³H]DNA preparation (86-90% for the single-copy [³H]DNAs used). Hybridization data were reduced by a nonlinear least-squares procedure (Pearson et al., 1977), assuming pseudo-first-order kinetics.

**RESULTS**

**Micromere Preparations**

Various stages in the isolation of micromeres are shown in Fig. 1. The final preparations, such as that illustrated in C, consisted of >99% micromeres. Preparations of
at least this degree of purity were obtained routinely by the procedures given in Materials and Methods and were used for all the experiments described subsequently.

Complexity of Micromere RNA

Total RNA was extracted from isolated micromeres, and its complexity was calculated from the amount of single-copy [3H]-DNA tracer hybridized at apparent kinetic termination. For comparison, reactions were also carried out with total RNA of 16-cell and 32- to 64-cell embryos. Figure 2 shows that micromere RNA hybridizes with 2.6% of the reactable single-copy DNA. The single-copy sequence content of the *S. purpuratus* genome is $6.1 \times 10^8$ nucleotide (nt) pairs (Graham et al., 1974), and given that single-copy sequences are represented asymmetrically in sea urchin RNAs (Hough et al., 1975; Lev et al., 1980), the complexity of micromere RNA is calculated to be about $3.2 \times 10^7$ nt. This is slightly lower than the complexity established for the total egg RNA of *S. purpuratus*, $3.7 \times 10^7$ nt (Galau et al., 1976; Hough-Evans et al., 1977). Though a relatively large amount of terminal data are presented in Fig. 2 for the micromere RNA reaction, there is no evidence for higher complexity components. The surprising implication is that micromeres lack detectable quantities of the high-complexity nuclear RNA observed previously in sea urchin embryos of later stages (Hough et al., 1975; Kleene and Humphreys, 1977; Wold et al., 1978; Ernst et al., 1979).

The hybridization experiments with 16-cell and 32- to 64-cell embryo RNAs shown in Fig. 2 demonstrate that a high-complexity micromere RNA component would easily have been noticed had it been present. RNA extracted from dissociated but unfractonated 16-cell embryos reacts with at least 8% of the single-copy DNA tracer. Therefore, an RNA whose complexity is significantly greater than that of egg RNA exists in the macromeres and/or meso-

meres of the fourth-cleavage embryo. This high-complexity RNA is almost certainly nuclear since previous studies have shown that in the 16-cell embryo, as in later embryos, the cytoplasmic RNAs are of equal or lower complexity than unfertilized egg RNA (Hough-Evans et al., 1977). RNA from 32- to 64-cell embryos also includes a very complex set of sequences. The 32- to 64-cell RNA hybridization reaction proceeds more rapidly, as might be expected since there are now more than two times as many nuclei in the same mass of cytoplasm. This is apparently due to an increase in the concentration of complex sequences in the total RNA. The dashed lines in Fig. 2 (see legend) represent the kinetics of these reactions calculated on the assumption that the complexity of the nuclear RNA in both the 16-cell and the 32- to 64-cell embryos is about the same as in later embryos, i.e., that the reaction terminates at 15%. However, this could not be explicitly demonstrated, since to do so would have required reactions to be carried out to $C_0t > 5 \times 10^6$ M sec.

Consider the situation that would result if each of the micromeres contained the same amount of high-complexity nuclear RNA as appears to be present (on the average) in the remaining cells. The four micromeres include only 7.5% of the cellular volume of the embryo, and their nuclei populate only about 3.3% of the total cytoplasmic volume (calculated from the dimensions of nuclei and cells in dissociated blastomere preparations). We assume that in micromeres the concentration of ribosomes per unit cytoplasmic volume is not significantly greater than elsewhere in the embryo. In this case the experiments of Fig. 2 would have revealed a high-complexity micromere RNA reaction occurring at a rate about 10 times faster than that observed with whole 16-cell embryo RNA. We observe a complexity equal to about 85% of that occurring in unfertilized egg RNA. Since no higher-complexity reaction could
FIG. 1. Micromere isolation. A 16-cell embryo (5.5 hr after fertilization) is shown in A. The fertilization membrane was removed 90 sec after the addition of sperm by a solution of 0.04% papain, 0.2% glutathione, pH 7.8. The hyalin layer is visible surrounding the embryo. Micromeres are indicated by the arrow (→). Embryos were dissociated in ice-cold calcium-magnesium-free seawater, and the resulting cell suspension is seen in B. Arrow indicates four micromeres. There are two macromeres above and two below the micromeres. At the X, four mesomeres can be seen above the upper two macromeres. C, taken at the same magnification, shows a preparation of isolated micromeres after Ficoll gradient separation. Scale bars represent 50 μm.

be detected, we conclude that there is a clear difference between the micromeres and the other cells of the fourth-cleavage embryo. The micromeres could contain—if any—no more than a few percent of the amount of high-complexity RNA found per
nucleus in the mesomeres and/or macro-

Maternal RNA Sequence Distribution

The purpose of the following experiments was to determine the fraction of the egg RNA sequence set present in micromeres and in micromere polysomal RNA. For these measurements, we utilized a single-copy $[^{3}H]$DNA tracer fraction ("egg DNA") which consists largely of sequences represented in unfertilized egg RNA. As described by Hough-Evans et al. (1977) and in Materials and Methods, egg DNA tracers were prepared by two successive rounds of hybridization of a single-copy tracer with egg RNA. In Fig. 3, the reactions of egg DNA with egg RNA and with micromere RNA are compared. Apparently only about 75% of the egg RNA sequence set is represented in the RNA of the micromeres. A similar result was reported by Rodgers and Gross (1978). Since the complexity of $S$. purpuratus egg RNA is $3.7 \times 10^7$ nt (Galau et al., 1976; Hough-Evans et al., 1977), the length of single-copy sequence shared by both egg and micromere RNAs is about $2.7 \times 10^7$ nt. This value is less than the total complexity of micromere RNA measured by reaction with single-copy DNA in the experiment of Fig. 2, i.e., $3.2 \times 10^7$ nt. If real, this discrepancy implies the existence of nonmaternal sequences in the micromeres. However, the difference is only $5 \times 10^6$ nt, close to the limit of resolution of these procedures. Some support for the possibility that micromeres possess a special set of single-copy RNA transcripts comes from the experiments of Rodgers and Gross (1978). They found that a tracer largely depleted of egg RNA sequences ("null egg DNA") reacts to about 1% with micromere RNA. This value implies independently that micromeres contain a nonmaternal set of RNA sequences of about $10^7$-nt complexity. However, due to the small magnitude of nonmaternal sequences in the micromeres. However, the difference is only $5 \times 10^6$ nt, close to the limit of resolution of these procedures. Some support for the possibility that micromeres possess a special set of single-copy RNA transcripts comes from the experiments of Rodgers and Gross (1978). They found that a tracer largely depleted of egg RNA sequences ("null egg DNA") reacts to about 1% with micromere RNA. This value implies independently that micromeres contain a nonmaternal set of RNA sequences of about $10^7$-nt complexity. However, due to the small magnitude...
FIG. 3. Hybridization of egg \([^{3}H]DNA\) with egg RNA, total micromere RNA, and polysomal micromere RNA. The solid curve shows reactions of egg DNA tracers with excess egg RNA (○). Egg DNA is a single-copy tracer consisting mainly of sequences complementary to egg RNA. Data were pooled from four egg DNA preparations by normalizing the average terminal value obtained with each preparation to 100%. These values ranged from 52 to 80% for the various tracers. Since only 3% of the starting single-copy tracer reacts with egg RNA, the egg RNA sequences in these preparations had been concentrated 17- to 27-fold. The pseudo-first-order rate constant of the solution shown is \(2.3 \times 10^{-4}\) M\(^{-1}\) set\(^{-1}\), as reported earlier (Hough-Evans et al., 1977).

The reactions of egg DNA with total micromere RNA (▲) and with polysomal micromere RNA (▼) are shown relative to the egg DNA-egg RNA reaction. The dashed line at 73% represents the reaction of a similar egg DNA tracer with total 16-cell embryo polysomal RNA (data originally presented by Hough-Evans et al., 1977).

The egg DNA was also hybridized with RNA extracted from the polysomes of isolated micromeres. These data are shown by the open symbols in Fig. 3. This result contrasts with the situation elsewhere in the fourth-cleavage embryo. Thus, Hough-Evans et al. (1977) showed that while the whole maternal RNA sequence set can be recovered from 16-cell embryo cytoplasm, only about three-fourths of these sequences are associated with polysomes. It follows that the micromeres lack the nonpolysomal maternal RNA sequences found in the cytoplasmic fraction of the whole embryo. In addition, though the evidence is not conclusive, micromeres may contain a small set of transcripts not found in the unfertilized egg. Such a sequence set would not have been detected in earlier studies on the complexity of 16-cell embryo RNA (Hough-Evans et al., 1977) because the micromeres contain too small a fraction of the embryo cytoplasmic RNA.

The most likely explanation of the lack of high-complexity nuclear RNA in the 16-cell micromeres is late activation of RNA synthesis. According to De Petrocellis et al. (1977), the first four divisions of the sea embryo. A brief summary of these differences follows: (a) Micromeres lack detectable quantities of the high-complexity nuclear RNA found in the macromeres and/or mesomeres, (b) micromeres contain only 75% of the set of maternal RNA sequences present in both the unfertilized egg and the cytoplasm of the whole 16-cell embryo; and (c) micromeres lack the nonpolysomal maternal RNA sequences found in the cytoplasmic fraction of the whole embryo. In addition, though the evidence is not conclusive, micromeres may contain a small set of transcripts not found in the unfertilized egg. Such a sequence set would not have been detected in earlier studies on the complexity of 16-cell embryo RNA (Hough-Evans et al., 1977) because the micromeres contain too small a fraction of the embryo cytoplasmic RNA.
urchin embryo (i.e., *Paracentrotus lividus*) are synchronous, but after this the micromeres divide at a slower rate. Autoradiograph and uptake experiments show that the first four micromeres carry out some RNA synthesis (Czihak, 1965; Hynes and Gross, 1970). The experiments of Fig. 2 show clearly that these cells do not contain typical concentrations of complex nuclear RNAs, as do the other cells in the fourth-cleavage embryo. It is important to note that until the 16-cell stage all the embryo nuclei synthesize RNA at a very low rate (perhaps 10%) relative to that observed in the 32-cell stage and thereafter (Czihak and Hörstadius, 1970; Wilt, 1970, personal communication). Thus, the induction of the “normal” embryonic rate of nuclear RNA synthesis may simply be delayed by one (or more) cell cycle in the micromeres, while it has already occurred in at least some of the fourth-cleavage macromeres and mesomeres.

Micromeres are essentially budded off with a very small amount of cytoplasm from the macromeres, and the key observation reviewed here may be that the micromere cytoplasm lacks the large set of nonpolysomal maternal RNAs found in other cells of the embryo. This suggests that the micromere cytoplasm constitutes a special domain distinct from the cytoplasm of the rest of the embryo. The micromeres may be the first set of cells in which the embryonic nuclei are exposed to a different environment, and this could affect the patterns of genomic expression in the micromere nuclei (i.e., transcription + processing). Thus the unique fourth-cleavage division by which the micromeres are segregated may provide the necessary condition for the establishment of the unique pattern of function their lineage later displays. Though not proven here, this probably means that the same subset of rare maternal messages is being translated in 16-cell micromeres as in mesomeres and macromeres.

Special regulators may be present in micromere cytoplasm which determine the micromere cell lineage. We would predict that significant differences in the micromere translational program will occur subsequently only as a result of the induction of a unique pattern of micromere nuclear RNA synthesis (and/or processing), rather than as the result of a regional fourth-cleavage localization of sets of maternal message sequences.

This research was supported by NIH Grant HD-05753. The sea urchin maintenance system was partially equipped with funds supplied by NIH Biomedical Research Support Grant RR-07003, and the culture system is maintained by NIH Grant RR-00986 from the Division of Research Resources. S.G.E. was supported by an NIH postdoctoral fellowship.

**REFERENCES**


ERNST ET AL. Micromere RNA Complexity


