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Nonrepetitive DNA Sequence Representation in Sea Urchin Embryo Messenger RNA

(^{3}H-labeled gastrula mRNA/hybridization/hydroxyapatite)

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ABSTRACT **Messenger RNA was prepared from developing sea urchin gastrulae by puromycin release from polyribosomes. Approximately 60% of the total mRNA radioactivity of the postnuclear supernatant was recovered and shown to be free of any other labeled RNA species such as ribosomal and nuclear RNA. The mRNA was examined by hybridization to DNA present in great excess. The mRNA hybridizes almost exclusively with nonrepetitive DNA. Almost all of the messenger RNA molecules of sea urchin gastrulae therefore consist of transcripts from nonrepetitive sequences. It appears that the structural genes expressed at this stage are typically not repeated in the genome and the mRNA does not include recognizable repetitive sequence.**

The DNA of higher cells is made up of repetitive and nonrepetitive sequences, many of which occur in an interspersed or alternating pattern (1). This pattern of organization is thought to determine the function of the genome and the regulation of genetic activity (2) and it is of considerable interest to establish experimentally the roles of the different sequences. The experiments reported in this paper were designed to determine whether the messenger RNAs of a developing animal system are transcribed from repetitive or nonrepetitive sequences. Previous work in other laboratories has indicated that certain specific structural gene mRNAs are probably transcribed from nonrepetitive DNA sequences, while histone gene sequences are probably repetitive (see ref. 3). Measurements of Greenberg and Perry (4) suggest that most L-cell mRNA is homologous to nonrepetitive DNA, while a small fraction may be transcribed from repetitive sequences.

MATERIALS AND METHODS

Growth of Embryos and Labeling of RNA. Strongylocentrotus purpuratus embryos were grown at 2×10^4 embryos per ml at 15° in Millipore-filtered sea water containing streptomycin and penicillin (5). They attained the 600-cell gastrula stage with three-pointed spicules by 36 hr. Between 36 and 39 hr they were labeled at 50 μCi/ml with [^{3}H]uridine (Schwarz-Mann, 26 Ci/mmol). 5 μCi/ml of $Na^{32}PO_4$ (New England Nuclear Corp., carrier-free) was added for the final 10 min of incubation to detect contaminating heterogeneous nuclear RNA (hnRNA).

Preparation of Nucleic Acids. DNA was extracted from sea urchin sperm by standard techniques, sheared to segments about 450 nucleotides long by passage through a press at 50,000 psi, and passed over Chelex 100 (Biorad, 200–400 mesh) (6).

RNA was extracted from messenger ribonucleoprotein (mRNP) preparations as described in the legend to Fig. 1 (*a–c*). The RNA was deproteinized with Sevag solution (24:1 chloroform–isoamyl alcohol) and phenol mixed 1:1. The mRNA was precipitated together with sheared sea urchin DNA and stored at −80° in distilled water.

mRNA·DNA Hybridization. mRNA and DNA were incubated at 60° (unless otherwise noted) in Kontes microflex vials in 0.12 or 0.41 M PB (PB is phosphate buffer that contains equal molar concentrations of Na_2HPO_4 and NaH_2PO_4). After incubation all hybridization mixtures were analyzed for possible RNA degradation by passage of an aliquot over a Sephadex G-100 column. Unless the elution pattern of the RNA was identical to the pattern before incubation, the sample was discarded. The hybrids were then analyzed in one of two ways. For the "RNase–Sephadex assay," the annealing mixture was brought to 0.24 M PB, incubated for 1 hr at room temperature with 10 μg/ml of ribonuclease (RNase A, Worthington), and passed over a Sephadex G-100 column. The amount of radioactivity in the exclusion peak refers to RNase-resistant hybrids. For the "urea–phosphate assay," the hybridization mixture was brought to 0.2 M PB, 8 M urea, 1% sodium dodecyl sulfate, and passed over a hydroxyapatite column (Biorad DNA grade HTP lot no. 9404) in the urea–PB solution at 40°. Single-stranded nucleic acids are not bound to the column in the urea–PB solution at 40°. The bound mRNA·DNA hybrids and DNA·DNA duplex are melted and eluted from the column at 80°. Measurements show that 10% less of the sea urchin DNA is recognized as repetitive by hydroxyapatite at 40° with 8 M urea and 0.2 M PB as compared to the standard 0.12 M PB 60° conditions (7).

RESULTS

Purity and Characteristics of the mRNA Preparation. To interpret the results of the mRNA·DNA hybridization experiments, the labeled mRNA must be free of other high-molecular-weight labeled RNAs such as hnRNA or rRNA.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; Pipes, piperazine-*N*-*N'*-bis 2-ethanesulfonic acid; PB, phosphate buffer; hnRNA, heterogeneous nuclear RNA; mRNP, messenger ribonucleoprotein.

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Since there is little rRNA labeling at the gastrula stage (8), our primary concern was contamination of the [^{3}H]mRNA preparation with [^{3}H]hnRNA. The embryos were labeled with ^{32}P during the final 10 min of the 3-hr labeling period. The only high-molecular-weight RNA that incorporates significant label during a 10-min period is hnRNA (9).

Fig. 1*a* illustrates the sedimentation of labeled polyribosomes in the postnuclear supernatant. In our experience relatively intact polysomal material can be obtained from *S. purpuratus* embryos only by the use of high ionic strength ($\geq$250 mM KCl), low pH (6.5), and ribonuclease inhibitors (polyvinyl sulfate and bentonite). The method of lysis described in the legend to Fig. 1 liberates 80–90% of the total cellular RNA and only about 10–20% of the ^{32}P-labeled hnRNA. Only the polysomes sedimenting at greater than 100 S (*bracket I*, Fig. 1*a*) were used for the subsequent steps. As shown in the figure most of the hnRNA was eliminated from the polysomal preparation. The polysomes were next treated with puromycin, which terminates protein synthesis and disaggregates the polysomal complex. After this treatment (Fig. 1*b*) polysomal mRNP and ribosomes sediment at less than 70 S (10). However, puromycin has no effect on the sedimentation of hnRNA. Thus this procedure eliminates the remaining hnRNA contamination as well as providing a functional criterion for the mRNA, i.e., sensitivity to puromycin release.

Approximately 60% of the mRNP appears to be bound to the ribosomal subunits. EDTA treatments of the <70 S material (*bracket II*, Fig. 1*b*) releases an additional 30–50% more mRNP from the subunits (Fig. 1*c*). Thus, 60–80% of

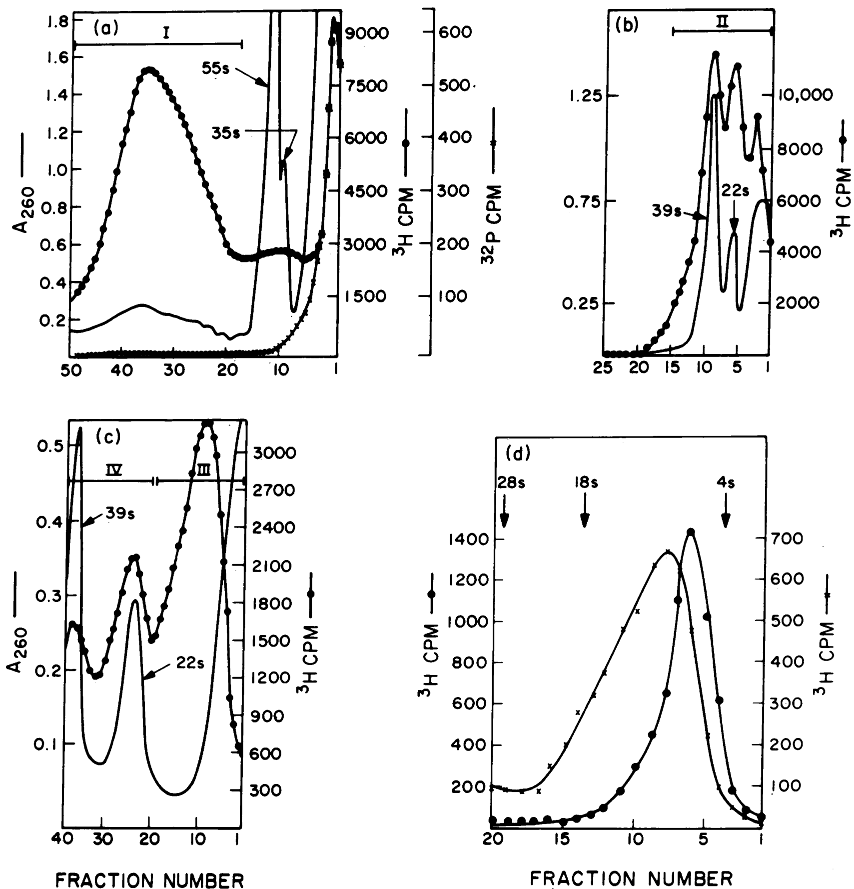

FIG. 1. Preparation of polyribosomes and purification of mRNA from sea urchin gastrulae. (*a*) Sedimentation of postnuclear supernatant in sucrose gradients. Embryos were grown and labeled with ^{3}H and ^{32}P as described in *Methods*. The embryos were harvested by centrifugation at 3000 × *g* for 5 min and washed at least three times with 1.5 M dextrose (22). They were resuspended in 50 mM Pipes buffer (pH 6.5), 250 mM KCl, 12 mM $MgCl_2$, 300 μg/ml of polyvinyl sulfate, and 5 mg/ml of bentonite to a concentration of approximately 4 × 10^5 embryos per ml. The embryos were lysed by 15 up-and-down strokes of a Dounce homogenizer (Kontes, 40-ml Dounce, B pestle). The lysate was centrifuged at 3000 × g to pellet nuclei, and Triton X-100 and sodium deoxycholate were then added to the postnuclear supernatant to a final concentration of 1% and 0.5%, respectively. Polyribosomes were prepared by centrifugation of the postnuclear supernatant through nonisokinetic exponential sucrose gradients. The following parameters as defined by Noll (23) were used to construct these gradients: V_m = 25 ml, C_t = 5%, and C_r = 61.5% (w/v) sucrose in 10 mM Pipes buffer (pH 6.5), 500 mM KCl, 5 mM $MgCl_2$, and 50 μg/ml of polyvinyl sulfate. The gradients were spun for 4 hr at 27,000 rpm in a Spinco SW 27 rotor at 4°, analyzed for absorbance by passage through a density gradient analyzer, and fractions were collected. An aliquot of each fraction was analyzed for trichloroacetic acid-precipitable radioactivity.

(*b*) Sedimentation of puromycin-treated polysomal material. *Fraction I* indicated in (*a*) was pelleted for 4 hr in a Spinco 60 Ti rotor at 60,000 rpm at 4°. The polysomal pellet was suspended in 50 mM Tris·HCl at pH 7.4, 1 mM $MgCl_2$, 50 mM KCl. The polysomes were disaggregated to mRNP and subunits by adjusting the polysomal buffer to 1 mM puromycin (Nutritional Biochemical Co.), 500 mM KCl, and by incubating for 30 min at 0° followed by 30 min at 37°. The puromycin-treated polysomes were sedimented through sucrose gradients similar to those described in (*a*).

(*c*) Sedimentation of the <70S puromycin-treated fraction after addition of EDTA. The <70S fraction [*bracket II* in (*b*)] was precipitated by adding ethanol to 50% and resuspended in 0.2 M EDTA at 15 A_{260} units of ribosomes per ml. The preparation was then centrifuged through isokinetic gradients at 50,000 rpm in a Spinco SW 50.1 rotor at 4° for 8 hr. The parameters employed (23) were: V_m = 6.14 ml, C_t = 15%, and C_r = 41.5% (w/v) sucrose in 10 mM Pipes buffer at pH 6.5, 50 mM KCl. For (*a–c*): A_{260} (——), [^{3}H]RNA (●——●), [^{32}P]RNA (×——×).

(*d*) Size of the isolated mRNA. *Fractions III* and *IV* of (*c*) were extracted for RNA as indicated in *Methods*. The mRNA was centrifuged in gradients similar to those described in (*c*) except that centrifugation was for 5 hr at 25° and the buffer for the sucrose was 0.1 M NaCl, 0.001 M EDTA, 0.5% sodium dodecyl sulfate, and 0.01 M Na acetate at pH 6.0. The positions of the sea urchin oocyte RNA markers are indicated by the *arrows*. RNA from *Fraction III*, (●——●); mRNA from *Fraction IV*, (×——×). The model S values of the mRNA preparations were 8 S for the *Fraction III* mRNA and 10 S for the *Fraction IV* mRNA.

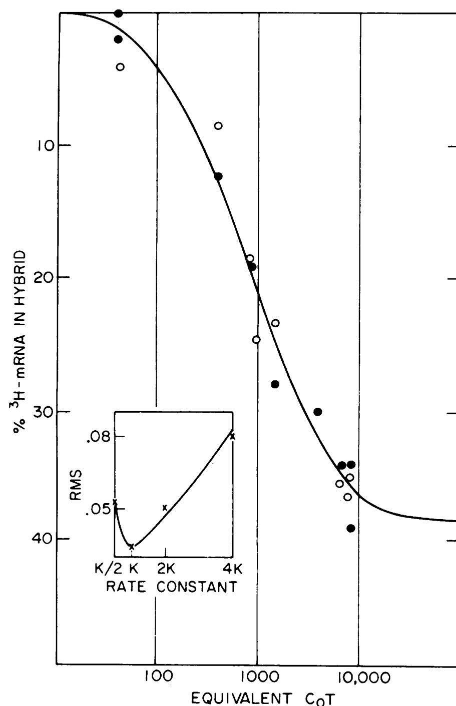

FIG. 2. Hybridization kinetics of mRNA to DNA. At least 100 μg of DNA and 10,000 cpm of [^{3}H]mRNA at DNA to RNA ratios of 1 to 2 $\times$ 10^4 were used in each determination. The mRNA·DNA hybrids were assayed by the RNase–Sephadex procedure (O——O) or the urea–phosphate procedure without RNase (●——●). For details of these two methods of analysis, see *Methods*. The solid line describing the rate of hybridization of mRNA to DNA was fit to the data points by a computer (6) using the rate constant $K = 0.00125$ measured for the reassociation of single-copy sequences in whole DNA (15). The *insert* represents a plot of root mean square errors (RMS) obtained as various other rate constants are fit to the data.

the total mRNP is freed from the ribosomal subunits by the combined treatment. mRNA was extracted from mRNP sedimenting at <20 S (*bracket III*, Fig. 1*c*) as well as that sedimenting with the ribosomal subunits at >20 S (*bracket IV*, Fig. 1*c*).

As illustrated in Fig. 1*d*, mRNA extracted from *region III* has a mean size of 8 S and a range in size of 4–17 S. mRNA extracted from *region IV* was somewhat larger, having a mean size of 10 S and a range in size of 4–27 S. The specific activity obtained for two mRNA preparations was 565,000 and 685,000 cpm/μg, on the assumption that 2% of the total polysomal 260-nm absorbance is due to mRNA (11).

In the final purified mRNA preparation, less than 0.2% of the ^{3}H radioactivity could be attributed to labeled hnRNA (Table 1). Because a portion of the mRNA preparation (that extracted from *fraction* IV, Fig. 1*c*) contained rRNA, it was important to determine the relative specific activity of the rRNA. From the relative rates of rRNA synthesis reported by Emerson and Humphreys (8) for this stage of development, it can be calculated that, under our labeling conditions, the specific activity for the embryo rRNA is only of the order of 500 cpm/μg. The elution pattern of the mRNA extracts on Sephadex G-100 also shows that less than 3% of the label could be in 5 S and tRNA. Therefore the specific activity of labeled nonmessenger RNA is too low to significantly affect the hybridization experiments.

Our data yield a value of 4×10^{-6} μg of newly synthesized mRNA per embryo accumulated during the 3-hr labeling period. If we accept the first order decay rate for sea urchin mRNA of 75 min reported by Brandhorst and Humphreys (12), the actual amount of mRNA synthesis occurring during 3 hr was about 1×10^{-5} μg per embryo. Considering the assumptions involved, this value is in fair agreement with the mRNA synthesis rates estimated by these authors (12).

Hybridization of mRNA to DNA at Repetitive C_0ts. The purified mRNA was hybridized with DNA present at 1 to 2 $\times$ 10^4 times the mRNA concentration to measure the DNA frequency components transcribed into mRNA (13, 14). By C_0t 40, most of the repetitive sequences in sea urchin DNA are reassociated, while less than 5% of the single copy sequences have reacted (15). To measure the fraction of total mRNA nucleotides which are transcribed from repetitive sequences, the amount of RNase-resistant mRNA·DNA hybrids which form by C_0t 40 was estimated by the RNase–Sephadex assay procedure. Measurements made in the absence of ribonuclease (urea–phosphate assay procedure) provide an estimate of the number of molecules containing recognizable repetitive sequence elements.

Measurements made by the urea–phosphate assay procedure are presented in Table 2. These data show that the repetitive sequence content of sea urchin gastrula mRNA is low or nonexistent. The amount of mRNA binding at C_0t 40 should be corrected for the small amount of mRNA binding in samples incubated to C_0t <0.001 and also for the 1–2%

TABLE 1. *Contamination of mRNA with hnRNA*

Fraction*	%[^{3}H]RNA as hnRNA by cpm ratio†	%[^{3}H]RNA as hnRNA by recovery‡
Postnuclear supernatant	41	36
Polysomes (I)	8	7
Polysomal pellet	0.7	0.8
20S mRNP (II)	0.15	0.14

* Roman numerals in parentheses denote fractions as illustrated in Fig. 1*a–b*.

† Calculated by using a ^{3}H to ^{32}P ratio of 6.0 for hnRNA. This ratio was determined in the following experiment: embryos were labeled with ^{3}H and ^{32}P for 3 hr and 10 min, respectively, and a preparation of nuclei was made as described in *Methods*. Chromatin was prepared from the nuclei and centrifuged to equilibrium in Cs_2SO_4 according to Wilt and Ekenberg (24). As described by these authors most of the hnRNA bands with a fraction of the chromatin which has a lighter buoyant density in Cs_2SO_4 than the majority of the chromatin. The ^{3}H to ^{32}P ratio in this region of the gradients was 6.0.

‡ An independent estimate of the [^{3}H]hnRNA contamination was derived from the maximum amount of the [^{3}H]hnRNA that could have been present in the starting >100S polysomes (fraction I of Fig. 1*a*) and the relative yields of [^{3}H]RNA and [^{32}P]-hnRNA at each stage of purification. The ^{3}H cpm in fraction 7 of the polysome profile presented in Fig. 1*a* are assumed to be due entirely to hnRNA. The maximum amount of hnRNA in the total [^{3}H]RNA is then estimated by summing the ^{3}H cpm that follow the aligned ^{32}P count profile.

mRNA hybridization with nonrepetitive sequences expected for C_0t 40. These corrections amount to about 3%, which is also the amount of mRNA binding obtained at C_0t 40 with calf-thymus DNA. Table 2 also shows that no additional repetitive transcript is recognized when the hybridization criterion is lowered to 50°. The measurements of Table 2 were performed at a DNA to RNA ratio of about 10,000, which supplies an excess of sites for all repetitive sequence hybridization.

We can conclude that, at most, 3% of the mRNA molecules contain repetitive sequence elements recognizable in the urea-phosphate assay. This small and somewhat uncertain degree of hybridization could be due to histone message (16, 17). The rate of histone synthesis, and thus the histone message quantity, is expected to be low at gastrulation since the rate of cell division is low (5, 18).

Hybridization Kinetics of mRNA. The rate of formation of mRNA·DNA hybrids can be calculated from the data of Fig. 2. The two sets of points are for urea–phosphate assay and the RNase–Sephadex assay. No significant difference is observed between the two sets of measurements.

The solid curve on Fig. 2 is the least squares solution for a single second order component with an assumed rate constant of 0.00125 M^{-1} sec^{-1}. This is the rate measured for a single copy fraction of *S. purpuratus* DNA (15), and is also the rate predicted for a genome the size of that of *S. purpuratus*. The *insert* on Fig. 2 shows that the lowest root mean square error is obtained with that rate constant. The increase in root mean square error for the other solutions is not so great, however, as to rule out rate constants of 0.0025 or 0.000625. Though two copies for each expressed gene sequence cannot be ruled out because of scatter in the data of Fig. 2, this seems a very unlikely possibility. While Melli *et al.* (13) suggest that the rate of DNA·RNA hybridization is one-half of the rate of DNA reassociation, Hutton and Wetmur (19) indicate that the rates are nearly the same. Our measurements lack the accuracy required to be applied to this issue. We conclude that there is probably only one copy in the DNA of sequences homologous to the mRNA. This conclusion is supported by measurements of the thermal stability, which show that the hybrid melts within 2–3° of reassociated single-copy DNA.

TABLE 2. *Hybridization of [^{3}H]mRNA with DNA*

DNA C_0t	% [^{3}H]mRNA hybridized
<0.001	4
40	5
40—50° reaction	4
40—calf thymus DNA	3
8000	33

Urea-phosphate assay of hybrids at DNA to RNA ratios of 1 to 2 × 10^4. The values represent the averages of six to eight individual determinations, except for the reaction at 50° (assayed at 30°) and the control in which calf thymus DNA was used. They were all carried out with mRNA preparations II, III, and IV (Fig. 1). The difference in the listed averages between C_0t <0.001 and 40 is 1%. The range of differences obtained in individual experiments varied from 0.8 to 2.5%.

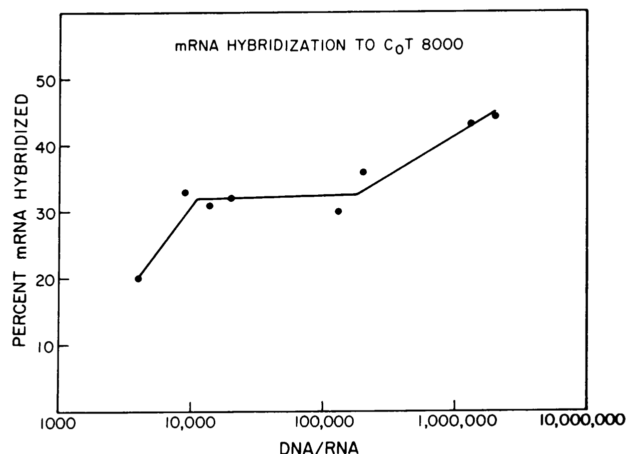

FIG. 3. Hybridization of mRNA to DNA at increasing DNA to RNA ratios. DNA and mRNA were annealed to a C_0t of 8000 and assayed without RNase treatment with the urea–phosphate assay procedure (see *Methods*).

The Effect of DNA to RNA Ratio on the Extent of Hybridization. The results presented in Table 2 and Fig. 2 were measured at DNA to RNA ratios of 10,000 to 20,000. The maximum extent of hybrid formation is about 35% of the total mRNA preparation at C_0t 8000, where the reaction is expected to be nearly complete. A possible explanation is that some mRNA species are present in so many copies that the homologous DNA sites were insufficient even at this ratio. Fig. 3 shows that as the DNA to RNA ratio is increased, additional hybridization occurs.

DISCUSSION

The fractionation procedure employed in this work yields a labeled mRNA preparation that is free from significant contamination by any other species of labeled RNA. The RNA which hybridizes in the experiments of Figs. 2 and 3 and Table 2 is therefore experimentally defined as messenger RNA. The yield in its preparation was about 60% of the labeled mRNA present in the post-nuclear supernatant. No selection of any particular class appears to have occurred as a result of losses during the preparation.

The hybridization behavior of the mRNA shows that it is transcribed almost exclusively from nonrepetitive sequences in the DNA. This finding provides direct evidence for the single occurrence (per haploid genome) of most or all of the large population of structural gene sequences which are expressed in gastrulation. Though only a small fraction of the structural genes are expressed at this stage of development, the sample is large enough to support the generalization that sea urchin structural genes are in general single copy sequences. Our results are similar to those reported by Kedes and Birnstiel (16) for the ">25S mRNA" (nonhistone) message of sea urchin embryos, although it is not completely clear that these authors were dealing with mRNA rather than with some other labeled species. Firtel *et al.* (20) have reported that the total mRNA of the slime mold *Dictyostelium discoideum* is transcribed primarily from single-copy sequences, and a similar observation has been made by Greenberg and Perry for L-cell mRNA (4). The one clear exception is provided by the histone genes, and others will probably appear. However,

it now seems safe to assume that few structural genes are repeated.

The urea–phosphate assay procedure provides a means of detecting repetitive elements on mRNAs even if they constitute only a small fraction of each mRNA molecule. Such a composite mRNA sequence organization is claimed to exist in *Xenopus* embryo mRNA by Dina *et al.* (21). Though our procedure would probably not detect very short repetitive sequences (<40 nucleotides), it is clear from the results presented in Table 2 that less than 3% of sea urchin mRNAs contain recognizable repetitive sequences. From the mRNA size distributions in Fig. 1*d*, it is likely that the final purified mRNA preparations have suffered an average of 1–3 strand scissions per molecule. However, if all mRNA contained a repetitive sequence element, a large amount of binding (>30%) should have been detected in the C_0t 40 hybridization experiments carried out in urea–phosphate. We have concluded that sea urchin mRNA molecules consist almost exclusively of nonrepetitive sequence transcript. Thus the transcripts (if any) of repetitive sequence regions adjacent to structural genes do not, in general, remain associated with the coding sequences on the polyribosomes.

From the results presented in Fig. 3 it is evident that different mRNA species must be present in differing numbers of copies in the gastrula. Complicating factors probably preclude a direct quantitative interpretation of Fig. 3. Calculations suggest that at extremely high DNA to RNA ratios the reaction may be impeded. It is clear, however, that practically all the information in the structural gene sequences expressed at gastrulation is present in the fraction of messages hybridizing at the lower DNA to RNA ratios.

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1. Davidson, E. H., Hough, B. R., Amenson, C. S. & Britten, R. J. (1973) *J. Mol. Biol.* **77**, 1–23.
2. Britten, R. J. & Davidson, E. H. (1969) *Science* **165**, 349–357.
3. Davidson, E. H. & Britten, R. J. (1973) *Quart. Rev. Biol.*, **48**, in press.
4. Greenberg, J. R. & Perry, R. P. (1971) *J. Cell Biol.* **50**, 774–786.
5. Hinegardner, R. T. (1967) in *Methods in Developmental Biology*, eds. Wilt, F. H. & Wessell, N. R. (Thomas Y. Crowell Co., New York), pp. 139–155.
6. Britten, R. J., Graham, D. E. & Neufeld, B. R. (1973) *Methods in Enzymology*, eds. Colowick, S. P. & Kaplan, N. O. (Academic Press, New York), in press.
7. Smith, M. J., Hough, B. R., Chamberlin, M. E. & Davidson, E. H. (1973) Submitted to *J. Mol. Biol.*
8. Emerson, C. P. & Humphreys, T. (1970) *Develop. Biol.* **23**, 86–112.
9. Wilt, F. H., Aronson, A. I. & Wartiovaara, J. (1969) in *Problems in Biology: RNA in Development*, ed. Hanley, E. H. (University of Utah Press, Salt Lake City), pp. 331–348.
10. Blobel, G. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 832–835.
11. Humphreys, T. (1971) *Develop. Biol.* **26**, 201–208.
12. Brandhorst, B. P. & Humphreys, T. (1972) *J. Cell Biol.* **53**, 474–482.
13. Melli, M., Whitfield, C., Rao, K. V., Richardson, M. & Bishop, J. O. (1971) *Nature New Biol.* **231**, 8–12.
14. Gelderman, A. H., Rake, A. V. & Britten, R. J. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 172–176.
15. Britten, R. J., Graham, D. E. & Henerey, M. (1972) *Carnegie Inst. Wash. Year B.* **71**, 270–272.
16. Kedes, L. H. & Birnstiel, M. L. (1971) *Nature New Biol.* **230**, 165–169.
17. Weinberg, E. S., Birnstiel, M. L., Purdom, I. F. & Williamson, R. (1972) *Nature* **240**, 225–228.
18. Kedes, L. H., Gross, P. R., Cognetti, G. & Hunter, A. L. (1969) *J. Mol. Biol.* **45**, 337–351.
19. Hutton, J. R. & Wetmur, J. G. (1973) *J. Mol. Biol.* **77**, 495–500.
20. Firtel, R. A., Jacobson, A. & Lodish, H. F. (1972) *Nature New Biol.* **239**, 225–228.
21. Dina, D., Crippa, M. & Beccari, E. (1973) *Nature New Biol.* **242**, 101–105.
22. Hinegardner, R. T. (1962) *J. Cell. Biol.* **15**, 503–508.
23. Noll, H. (1967) *Nature* **215**, 360–363.
24. Wilt, F. H. & Ekenberg, E. (1971) *Biochem. Biophys. Res. Commun.* **44**, 831–836.