TRANSLATION-LEVEL CONTROL OF PROTEIN SYNTHESIS DURING EARLY DEVELOPMENT[1]

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There is increasing support for the idea that protein synthesis in cells of multicellular organisms may be regulated at the level of translation. Control of this kind is assumed to have been demonstrated when a normal pattern of protein synthesis develops in the absence of gene transcription. Experiments with Actinomycin D show that the initiation of protein synthesis at fertilization in sea urchin eggs is independent of simultaneous gene action, and that a normal rate of protein synthesis is maintained up to the blastula stage without new information from the genome (Gross, Malkin, and Moyer, 1964). It was suggested early (Gross and Cousineau, 1963) that some of the embryo's protein synthesis occurs on messenger RNA made and stored during oogenesis. Evidence favoring such a scheme has continued to appear; e.g., synthesis of stable heterogeneous RNA prior to fertilization in the egg of Xenopus (Brown and Littna, 1964), a demonstration of translation-level control of globin synthesis in the chick embryo (Levere and Granick, 1965), and a proposal of Spirin and Nemer (1965) that "old" and "new" polyribosomes in the developing sea urchin are distinguishable, the old ones being exclusively active in protein synthesis during early stages.

It now becomes important to ask what kinds of proteins are made on the maternal messenger RNA, and what changes in pattern are due to new messengers, which appear to be synthesized at an increasing rate throughout early development (Gross, Kraemer, and Malkin, 1965). Stated this way, the question concerns the whole pattern of protein synthesis, rather than changes in a few species selected for their ease of assay. One is thus confronted

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with the technical problem of analyzing a very large population of soluble proteins and detecting newly-synthesized species therein.

Recently, Spiegel, Ozaki, and Tyler (1965) reported the results of a study in which soluble sea urchin embryo proteins were separated by electrophoresis on polyacrylamide gels that were then sliced transversely and eluted for counting. We have been engaged in similar work, using several methods including gel electrophoresis. For analysis of radioactivity distribution in the gels, however, we have employed the method of longitudinal slicing and autoradiography developed by Fairbanks, Levinthal, and Reeder (1965). This technique offers greatly improved resolution, since there is no need to cut fractions of arbitrary size from the separation gel, a procedure that must degrade the quality of the separation achieved by electrophoresis.

Our results agree on some points with those of Spiegel, et al. (1965), but the improved resolution of radioactive bands offered by the autoradiographic method has allowed the detection of significant changes in the pattern of protein synthesis not previously observed. These changes, and their course in Actinomycin-treated embryos, have an important bearing on the relations between protein synthesis and differentiation. We report here several of the major qualitative findings. A more complete description and analysis of these and related experiments will be published elsewhere.

Methods: Eggs of Arbacia punctulata were washed (before and after fertilization) and incubated in sterile sea water containing penicillin and streptomycin as described earlier (Malkin, Gross, and Romanoff, 1964). Development took place in the dark at 21.5°C, with gentle agitation at 70 oscillations per minute. Embryos to be treated with Actinomycin were exposed one hour before fertilization and continuously thereafter, at a concentration of 20 μg per ml. The Actinomycin-treated embryos hatched as blastulae ten hours after fertilization, an overall delay of about 10% with respect to controls. Morphogenetic events in Actinomycin D have been described in an earlier paper (Gross and Cousineau, 1964).

At various times during development, 5×10^5 embryos were removed and washed with sterile sea water containing appropriate antibiotics. They were then suspended in 12 ml of the same medium containing ^{14}C-algal hydrolyzate (reconstituted, Schwartz BioResearch), with the amino acids at an average specific activity of 125 mc/m mole and a total activity in the medium of 4 to 8 μc/ml. Incorporation was stopped by washing the embryos in ice-cold Mg^{++}-Ca^{++}-free sea water containing 1% casamino acids. Cells were packed by centrifugation, quick-frozen, and stored at -15°C.

For protein anaylsis, the cells were thawed and suspended in cold buffer (0.01 M, Tris, pH 7.4; 0.01 M KCl; 0.015 M $MgCl_2$) and homogenized with a

tight Dounce homogenizer. Homogenates were centrifuged for 25 minutes at 18,000 X g, and the supernatants were then centrifuged for 5 hours at 105,000 X g, all at 2°C. Aliquots of the final supernatants were used for electrophoresis.

Disc electrophoresis in basic gels was carried out essentially as described by Davis (1964). The 6 mm i.d. pyrex tubes contained 1.9 ml of 10% polyacrylamide in the small-pore lower gel, 0.8 ml of 2.5% stacking gel, and 0.35 ml protein sample with 3-7 mg protein and 0.5-1.3 X 10^6 TCA-precipitable cpm. The samples were saturated with sucrose and covered with 0.1 ml of 2.5% gel as anticonvection measures. Electrophoresis took place at 4°C, with constant current of 4 ma/tube. Arbacia eggs contain a pigment which migrates at the same velocity as the usual tracking dye. Electrophoresis was therefore continued until the pigment band reached the bottom of the separation gel, and no tracking dye was used.

Gels were removed from the tubes and stained with Amido Black, destained by diffusion in 7% acetic acid, photographed intact, then sliced longitudinally, dried, and stored in contact with Kodak No-Screen X-Ray film. Positive transparencies of the stained gels, at 1 : 1 magnification, and the autoradiograms themselves, were scanned and recorded on a Joyce-Loebl microdensitometer.

Four to six stages of development were studied in this way in several separate experiments, with systematic variation of the extraction buffers and of separation gel concentrations. We report below results for which all experiments agree, although for brevity the data shown are confined to two stages of development.

Results: 1. The stained gels show about 20 prominent bands and a diffuse region that probably represents a large number of proteins migrating at nearly the same velocity (labeled Σ in figure 1). The patterns are almost identical from fertilization through gastrulation, and those from Actinomycin embryos are identical with the controls. There are some small changes, e.g., the density of the fastest band ("a" in fig.1) decreases with time. At the pluteus stage, alterations from the early pattern are more easily detectable. Thus through most of early development, changes in the distribution of radioactivity emerge on an essentially invariant distribution of bulk soluble proteins. Samples of stained gels from control 2-cell and gastrula stages are shown in fig. 1 (patterns no. 1 and 6, respectively). A densitometric tracing of the stained gel from control zygotes is shown in fig. 2.

2. The radioactivity patterns are complex, even early in development. Some radioactive species migrate in coincidence with stained bands and other ones do not. The autoradiogram for gel no. 1 is labeled "2", and is placed

alongside the stain pattern for comparison. "b" is a band of radioactivity for which there seems to be no stained counterpart. This relation is seen in fig. 2, the tracing of a zygote autoradiogram superimposed on the stain pattern.

3. During the period of cleavage, the autoradiographic patterns for Actin-

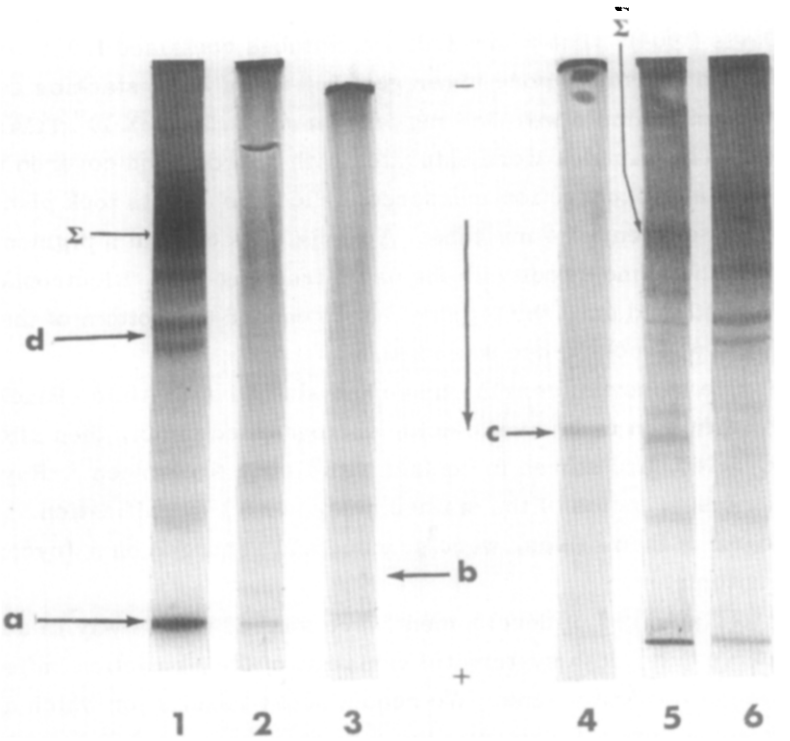

Figure 1. Stained band patterns and autoradiograms from electrophoresis of Arbacia soluble proteins. 1 and 6 are stain patterns from 1 hour (2 cell) and 18 hour (gastrula) control embryos. "a" is the fastest (anodically) migrating component. "d" is a bulk doublet in which little radioactivity is detected during cleavage, but which becomes more radioactive later. 2 is the autoradiogram obtained from a 1-month exposure of a dried slice from 1. 5 is similarly obtained from 6. The symbol Σ is explained in the text. 3 is an autoradiogram from proteins of Actinomycin embryos corresponding in time to 2; 4 is from an Actinomycin culture corresponding to 5. "b" is an early radioactive band that has no bulk counterpart, and "c" is a band that increases in prominence with time, both in controls and in Actinomycin. Exposure to labeled amino acids was for 20 minutes (2, 3, and 5) or 40 minutes (4).

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omycin embryos are essentially the same as those from controls. This is represented by gel no. 3, in fig. 1.

4. The manner of distribution of total grain density over length in the control autoradiograms changes with stage, i.e., patterns of radioactivity incorporated into proteins change in the course of normal development. The density distribution in autoradiograms tends ultimately to approach that in the stain

patterns. The behavior of the doublet "d", in fig. 1, is an example of this. A qualitative assessment of the extent of the change from zygote to gastrula can be made from fig. 1.

5. The density distributions in autoradiograms from Actinomycin-treated embryos also change with time. Some of these changes follow those taking place in the controls (e.g., the increasing prominence of the band "c"). Up to the time of hatching, Actinomycin and control patterns are quite similar. After hatching, control and Actinomycin patterns continue to change, but no longer in the same way (compare, for example, the appearance of the region marked Σ in gels 4 and 5, fig. 1).

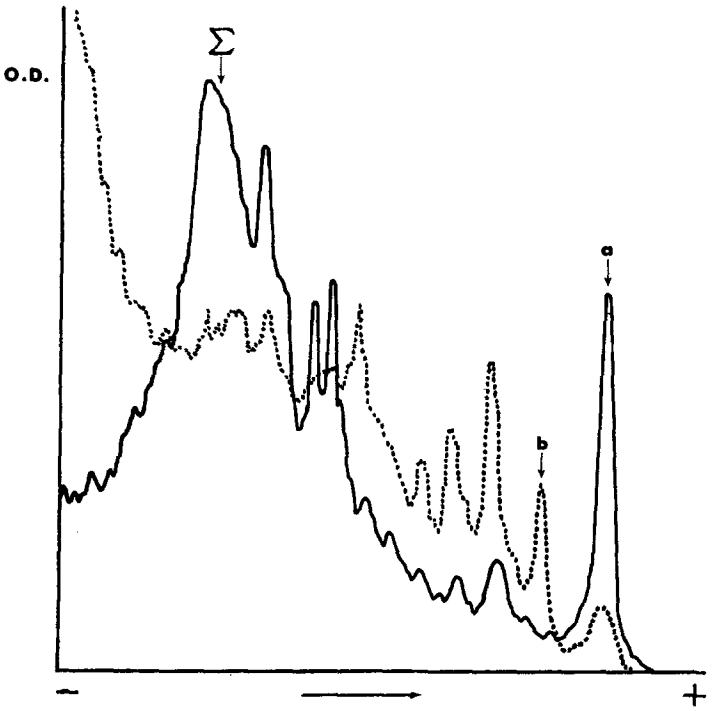

Figure 2. Microdensitometric tracing of the stained gel (continuous line) from zygotes, and superimposed, the tracing of the autoradiogram from a longitudinal slice of the same gel (broken line). The symbols are defined in fig. 1. Full-scale O.D. for the stain pattern was 2.0, and for the autoradiogram it was 0.5.

Discussion: These results agree with the ones reported by Spiegel, et al. (1965) in an important point, i.e., that radioactivity in newly-synthesized proteins follows a similar pattern in control and Actinomycin-treated embryos during the period of cleavage. Since Actinomycin inhibits the synthesis of new RNA with potential template function (Gross, et al., 1964), this is additional

evidence for the presence of a stored program for protein synthesis in the unfertilized egg.

We have, however, been able to detect, even with a less than satisfactory method for separating the proteins in the complex mixture extracted from the embryos, systematic changes in the pattern of protein synthesis over the time of normal development. These changes are evident in a comparison of the radioactivity distributions on zygote and blastula autoradiograms, to the extent, at least, that different ways of partitioning the total grain density over a fixed length of gel imply either differences in the kinds of protein being synthesized or changing rates of synthesis of species in the same set. The changes are obvious at gastrulation. Such alterations in the pattern of synthesis are not unexpected if differentiation depends upon differential gene action and protein synthesis, whereas a truly constant pattern of protein synthesis over the entire course of early development would have been surprising. The fact that Actinomycin embryos follow the controls with respect to a changing pattern of protein synthesis is not necessarily predictable, however. This result suggests that translation-level control of protein synthesis may be concerned not only with permitting the function of all messages, but perhaps also with differential rates or times of utilization.

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