SYNTHESIS OF SPINDLE-ASSOCIATED PROTEINS IN EARLY CLEAVAGE

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Synthesis of mitotic spindle proteins is probably one of the normal preparations for division in higher cells (Mazia, 1961). Not so clear, however, is the situation in early embryonic development. The most widely accepted point of view, based upon several independent lines of evidence, is that the unfertilized egg contains a store of preformed spindle precursors, and that the controlling process is assembly, rather than biosynthetic elaboration, of the working mitotic apparatus (MA). The evidence is very thoroughly reviewed in Mazia's monograph (1961).

Protein synthesis begins in sea urchin eggs immediately after fertilization (Nakano and Mon-

roy, 1958; Hultin, 1961; Gross and Cousineau, 1963 *a*). There is, however, no net increase in protein during early development; on the contrary, total protein declines (Kavanau, 1953). The synthesis of some protein or proteins is essential to division, since treatment of the fertilized eggs with Puromycin blocks amino acid incorporation *and* mitosis in any cleavage cycle (Hultin, 1961). Recent experiments with Actinomycin D (Gross and Cousineau, 1963 *b*) show that division and protein synthesis survive treatment with the antibiotic, while RNA synthesis and differentiation are quickly brought to a halt. There is a differential response of ribosomes from fertilized and unfertilized eggs to stimulation by poly-U (Nemer, 1962; Wilt and Hultin, 1962; Tyler, 1962), and Denny (1963) has reported that artificially activated, non-nucleate egg fragments are also stimulated to incorporate labeled amino acids into protein.

We have, with these observations in mind, suggested (Gross and Cousineau, 1963 *b*) that the unfertilized egg may contain a store of blocked messenger RNA which is activated and acquires the power to combine with ribosomes after fertilization; that the program of some of these messages is, furthermore, concerned with division proteins.

The proposed "division proteins" need not be structural components of the mitotic apparatus; it would be surprising if at least some of them were not histones and other nuclear proteins. But the possibility of *some* synthesis of structural or enzymic elements, concerned with the spindle itself, is not ruled out by available evidence which identifies macromolecular "spindle precursors." The autoradiographic observations described here suggest that such synthesis does occur.

Eggs of *Arbacia punctulata* were fertilized normally in sea water containing DL-leucine-H^3, at a final concentration of 0.47 mμM/ml (5.4 c/mM). The cultures were sampled at intervals during the early cleavages by fixing the cells in acetic-alcohol (1:3). Fixed eggs and embryos were processed by ordinary histological techniques, and ultimately sectioned at 5 μ. The sections, after removal of paraffin, were washed with cold 5 per cent TCA and then very thoroughly with water; they were finally dipped in Kodak NTB2 nuclear emulsion. After drying, and exposure periods of 1 to 3 days, the autoradiograms were developed and stained through the emulsion with Azure B.

Beyond the 8-cell stage, the embryos are very heavily labeled, and the autoradiograms show an essentially uniform distribution of grains over cytoplasm and nuclei. But the early cleavages show a strikingly different pattern, which is represented by the photomicrographs in Fig. 1. Photograph *a* shows the usual condition observed at the time the spindle is beginning to form (*i.e.*, after the "streak" stage and just before definitive prophase). There are silver grains over the entire cell, including the nucleus, but an obvious concentration is manifest over the forming spindle, appearing in these sections as a more densely and uniformly stained fusiform region surrounding the nucleus. This pattern is seen in all sections at the same stage, so that the grain distribution described is not accidental. Earlier stages have more uniformly distributed radioactivity. The peripheral cytoplasmic grains are likewise not accidental; sections such as those shown are 30 to 60 μ in diameter, so that peripheral grains are not caused by more central decay processes; the range of tritium β^- particles is not sufficiently long in the emulsion. At a slightly more advanced stage, the "prespindle," which consists of developing asters and some fibrous elements of the spindle proper beginning to push in on the nucleus, is easily distinguished, by both its staining and a more obvious localization of radioactivity. This is represented by Fig. 1 *b*. At metaphase, shown in Fig. 1 *c*, almost all of the radioactivity is found over the mitotic apparatus.

The second and third division show similar phenomena, except that the total radioactivity rises steadily and a greater proportion of the emulsion grains remain in the peripheral cytoplasm. A two-cell embryo in prophase of the second division is shown in Fig. 1 *d*; the lower blastomere is sectioned through the nucleus, while the upper contains only the "prespindle." Central localization of the radioactivity is evident.

An instructive exception to the centralization of radioactivity during mitotic stages is seen in eggs blocked at prophase by immersion in D_2O (see Gross and Spindel, 1960, Marsland and Zimmerman, 1963). In such eggs, a diminished "prespindle" remains indefinitely condensed about the nucleus. These eggs, however, also form large numbers of cytasters, which are capable of mitotic work, since upon the removal of D_2O they engage chromosomes released at nuclear breakdown, forming several multipolar spindles in each cell; furrows then dip down simultaneously between

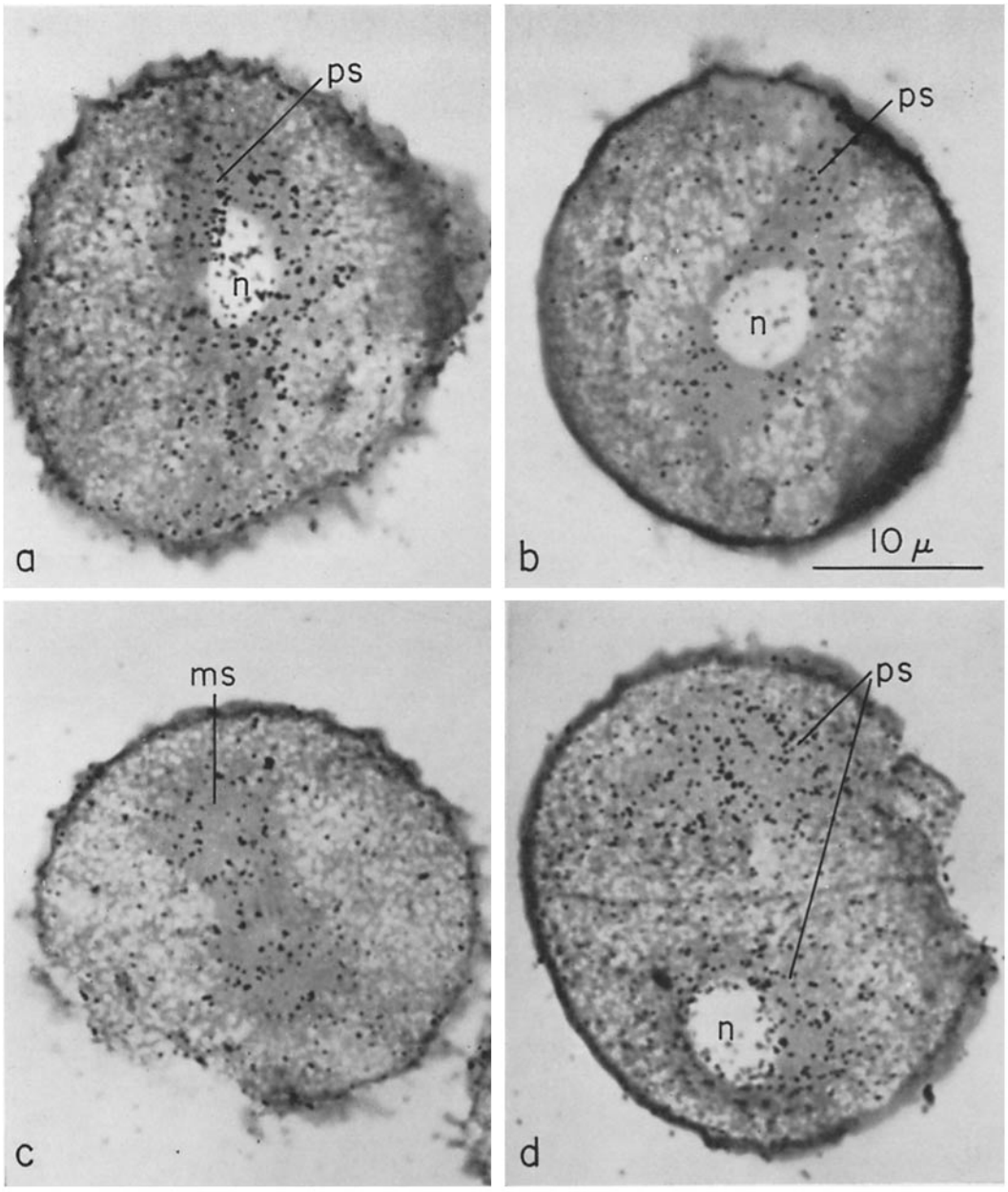

FIGURE 1 Autoradiograms of sea urchin eggs during the first two cleavages. Continuous incorporation of DL-leucine-H^3 (5.4 c/mM) at a concentration of 0.47 mμmole/ml in the medium. *a*, *b*, and *c*: first division; early prespindle, prophase, and metaphase, respectively. *d*, prophase of the second division. *ps*: prespindle; *ms*: metaphase figure; *n*: nucleus. Silver grains identify radioactivity incorporated into newly synthesized protein. Kodak NTB-2 emulsion, exposed for 3 days. Sections stained through the emulsion with Azure B.

pairs of adjacent cytasters. During the D_2O blockade, the eggs do not replicate their DNA (Gross and Harding, 1961), but they do incorporate labeled amino acids into protein. An autoradiogram of such a cell labeled with tritiated leucine is shown in Fig. 2, with cytasters outlined in the inset. These cells show silver grains over the nuclei and the perinuclear "prespindle," but

the cytasters are also labeled, at the periphery. Indeed, there is usually *no* label elsewhere, when the contribution of background is considered.

The localization of radioactivity in elements of the mitotic apparatus is most easily interpreted as reflecting synthesis, during interphase, of some protein or proteins playing a structural or catalytic role in the operation of the spindle. There are, however, a number of possible alternatives which must be considered. Some of the most likely of these, and the reasons for discarding them, will be mentioned here, but a full discussion will be reserved for another place.

The differential staining of the prespindle and its electron microscopic image agree in showing a high density of ribosomes therein (see, *e.g.*, Gross, Nass, and Philpott, 1958; Harris, 1962). It is likely, in fact, that the number of ribosomes per unit area of section is greater in the spindle region than in more peripheral parts of the cell. The larger formed elements of the cytoplasm (pigment granules, yolk particles, mitochondria, fat droplets) are extruded from the region of the growing prespindle, but ribosomes are not. Thus, while the interstices between formed elements in the peripheral cytoplasm are characterized by a high density of ribosomes, these particles, together with vesicles, tubules, and fibers (as shown by Harris, 1962) are the *only* visible components of the sectioned prespindle.

The first alternative explanation for the incorporation pattern with labeled amino acids is, therefore, that the label is in nascent, ribosome-bound protein. Since there are more ribosomes

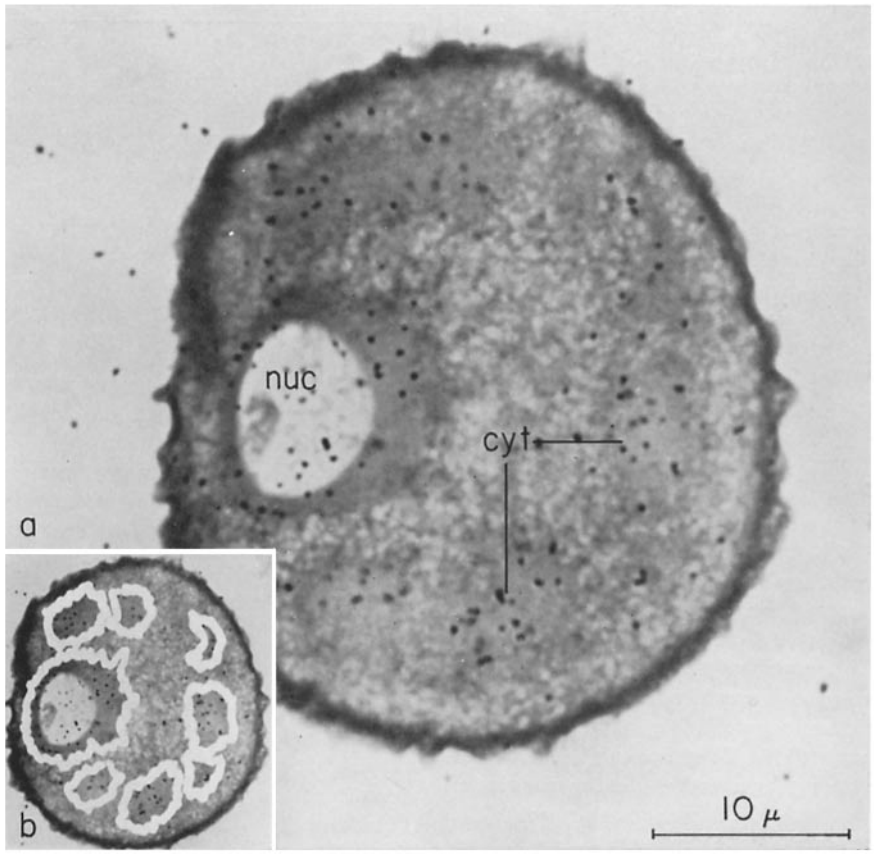

FIGURE 2 *a*. Autoradiogram of section of a sea urchin egg blocked in prophase of the first cleavage division by immersion in 90 per cent D_2O-sea water. Inset (*b*) shows location of the prespindle (perinuclear area outlined in white) and of the cytasters (peripheral areas outlined in white). Labeling with DL-leucine-H^3 as in experiment represented by Fig. 1. *nuc:* nucleus; *cyt:* cytasters. × 2,700.

per unit area in the prespindle, there will be a larger number of grains over this region; but the labeled proteins need have nothing to do with mitosis. We regard this proposal as unlikely, since the exposure to label was continuous, and the shortest incubation interval before fixation was 40 minutes. We have no data concerning the time necessary for the completion of an average protein on sea urchin egg ribosomes, but on the basis of reports concerning this interval in other higher cells (see, *e.g.*, Warner *et al.*, 1963), which is found to be closer to 40 *seconds*, we may assume that most of the silver grains seen in the autoradiograms represent completed protein, and are hence not labels for ribosomes.

Again, it might be proposed that the many ribosomes trapped in the spindle are activated, perhaps by nuclear messenger RNA, and that their product, which has nothing to do with mitosis, is sequestered and escapes to a general distribution only after the mitotic apparatus has broken down. This is made unlikely by the return of radioactive localization in mitotic figures after the first, and by the length of interphase, during which incorporation occurs, relative to the mitotic stages.

A third alternative is that the radioactivity is due to spill-over and trapping of newly synthesized proteins from the nucleus. But this is unlikely because the nuclei are not more heavily labeled than the prespindle; indeed, they are usually less radioactive (see Figs. 1 *b* and *d*, and 2). The labeling of the cytasters (Fig. 2) also eliminates this explanation.

There is finally (though it does not exhaust the alternatives) the important point concerning quantity, *i.e.*, stated as an objection, that not enough protein is made to contribute significantly to the structure of the mitotic apparatus, which contains at metaphase, according to Mazia and Roslansky (1956), about 12 per cent of the cell's protein. This value was obtained from analysis of the isolated MA. It may also be recalled that total protein declines somewhat during early development (Kavanau, 1953; Gustafson and Hjelte, 1951).

Data necessary for an estimate of the true rate of synthesis during early cleavage are not available, but a rough minimum estimate can be made from bulk incorporation experiments. Fertilized eggs were incubated with C^{14}-L-valine at two different specific activities; a uniformly labeled product at 205 mc/mM and one labeled in C-1 to 5.7 mc/mM. Final concentrations in the incubation medium were 0.23 mμM/ml and 40.7 mμM/ml, respectively. Entry of the amino acid is probably passive, for incorporation of label into proteins was proportional to concentration. The observed rates are thus a function of the concentration gradient across the membrane. Since we have no guarantee that the lower specific activity (and higher concentration) was sufficient to saturate, the protein synthesis rate estimated from valine uptake must be less, probably much less, than the true rate.

Some results from these experiments are shown in Table I. Using Kavanau's (1953) data for the valine content of total egg proteins in *Strongylocentrotus purpuratus*, we compute minimum turnover

TABLE I

Incorporation of C^{14}-L-Valine Into Protein of Dividing Sea Urchin Eggs

Specific activity of precursor	Final concentration of valine sea water	Valine incorporated by 10^6 cells		Cumulative synthesis (calculated) Total protein in unfertilized egg	
		mM × 10^5		*per cent*	
		End of 1st div.	End of 2nd div.	End of 1st div.	End of 2nd div.
mc/mM	*mμM/ml*				
5.73	40.7	5.71	10.5	0.17	0.31
205	0.23	0.144	0.267	0.004	0.008

for proteins of "average" composition as 0.17 and 0.31 per cent, relative to the cellular total, at the end of the first and second cleavage cycles, respectively. The valine content calculated from Kavanau's data is high, 10.7 per cent. If the valine content of the proteins being assembled is closer to normal (*ca.* 6 per cent), the calculated synthesis rate would be correspondingly higher.

These values are certainly not in agreement with the observation that the isolated metaphase figure contains 12 per cent of the total protein, if one assumes that the isolated MA is composed entirely of mitotic machinery. But the electron microscope reveals that the fibrous component of the MA accounts for only a small fraction of its volume (Gross *et al.*, 1958; Harris, 1962). The isolated MA is probably as much a *region* of the cell as it is an organelle. Also, 40 per cent of the volume of an egg is occupied by formed elements (Costello, 1939); hence, the 12 per cent figure means that a much larger proportion of the ground substance

protein is held in the region of the spindle. This implies that synthesis of a small amount of protein in the cytoplasmic matrix could produce the working fibrous component, and that a very much larger quantity of protein from the ground cytoplasm could be enmeshed in its vicinity.

It follows, therefore, that neither the amount of protein made during early cleavage nor the autoradiographic localization of the product is inconsistent with synthesis of important proteins for the mitotic apparatus. The synthesis of such molecules could provide a primary channel for the regulation of cell division.

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