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*ACCUMULATION IN THE OÖCYTE NUCLEUS OF A GENE PRODUCT ESSENTIAL FOR EMBRYONIC DEVELOPMENT BEYOND GASTRULATION**

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During the past several years a number of genes have been discovered by Humphrey in the Mexican axolotl—genes that affect development at various times ranging from oögenesis to larval stages.[1-8] Of these, the ones that exert effects on the egg cytoplasm during oögenesis are of special interest because of the possibility that they may eventually provide new ways of studying the egg cytoplasm and its control over the early development of the embryo. One gene in particular seems promising in this regard.[9] This gene, designated by the letter *o* because of its effect on the oöcyte cytoplasm, acts as a simple recessive. Heterozygotes (+/*o*) are indistinguishable from wild-type individuals. When mated with each other, they produce offspring all of which develop normally through embryonic and early larval stages. The homozygous (+/+) and heterozygous (+/*o*) individuals from such matings continue to develop normally throughout. The homozygous recessives (*o*/*o*), on the other hand, begin to display certain abnormalities during larval life. These include a somewhat slower than normal growth rate, and a marked reduction in the capacity to regenerate amputated limbs.[9,10] Nonetheless, these *o*/*o* individuals from heterozygous parents eventually attain sizes approaching that of full-grown normal axolotls, and appear to function normally or nearly so with one important exception—that of reproduction. About one half of the *o*/*o* animals remain juvenile with respect to secondary sexual characteristics and on further analysis are shown to be sterile males. The remaining half of the *o*/*o*'s appear to be normal females, but when mated with +/+ or +/*o* males, they spawn fertilized eggs of a unique type. Regardless of the genotype of the sperm, these eggs develop normally to blastula stage but are then without exception arrested during gastrulation, usually at a stage not more advanced than the crescentic blastopore stage. In relatively rare spawnings the blastopore closes completely and the embryos elongate, but even in these cases there is no evidence of neural fold formation. On the basis of this evidence it appears that gene *o* exerts a maternal effect,

modifying the egg cytoplasm during oögenesis in such a way as to lead to a cessation of development considerably later on, just prior to the time when the main axial organs of the embryo are being laid down.

In view of its possible importance as a tool in analyzing the morphogenetic organization of the egg cytoplasm, we have undertaken a series of studies on the maternal effect exerted by gene *o*. The experiments to be reported in this paper are of three types. (1) We have repeated Humphrey's study of the eggs of *o/o* females and have found, as he did, that these eggs are invariably arrested prior to neurulation, whether or not the normal allele of gene *o* is introduced by the sperm at fertilization. (2) The possibility that the maternal effect of gene *o* is in the nature of a cytoplasmic deficiency has been tested by injecting cytoplasm of mature normal (+/+ or +/*o*) eggs into fertilized eggs of *o/o* females. Such injections lead to a striking improvement in the development of the recipient eggs. (3) Finally, the localization of the corrective material, presumably a product of the normal allele of gene *o*, has been studied in normal ovarian eggs at a time when the large egg nucleus (germinal vesicle) is still intact. The corrective material is found in much higher concentrations in the nuclear sap than in the cytoplasm of such eggs.

Development of Untreated Eggs of o/o Females (Table 1).—Our observations on these eggs confirm those of Humphrey.[9] Cleavage is normal at first, and development proceeds to mid- to late blastula stages before the first signs of the maternal effect of gene *o* become apparent. At this time the cell division rate slows down so that cell size comes to be noticeably larger than it is in normal embryos of comparable age. Also, the embryos at this stage usually display a transient "dotted" appearance, resulting from an accentuated accumulation of melanin granules around the cell nuclei, especially in the animal hemisphere. In some spawnings the embryos are arrested in late blastula stage, but in the majority the embryos form the dorsal lip of the blastopore and proceed partly through gastrulation before development comes to a stop. In our experiments the embryos never developed beyond mid-gastrula stage. Humphrey has observed, in a small minority of spawnings, that the blastopore may close completely and the embryos elongate somewhat before development is arrested.

TABLE 1

EFFECT OF CYTOPLASM FROM NORMAL MATURE EGGS ON THE DEVELOPMENT OF EGGS OF *o/o* FEMALES

Material injected	No. of eggs injected	No. of complete blastulae	Development of Blastulae: Arrested blast. and gast.	Correction of Maternal Effect of *o*: Gastrulae	Neurulae	Postneurulae
Control (nothing injected)	0	1208	1208			
Cytoplasm from eggs of normal females	267	186	4	6	29	147

1. Eggs of 27 *o/o* females were used in the experiments summarized in this table. Thirteen of these were mated with +/+ males, the other fourteen with males that could have been either +/+ or +/*o*. The eggs, when untreated, were all arrested at blastula or gastrula stages, regardless of possible differences in the genotype established at fertilization.

2. Cytoplasm transfers were performed in Steinberg's solution,[12] pH 7.4, containing penicillin (0.01%), streptomycin (0.01%), and Elkosin, Ciba (0.05%). Both experimental and control eggs were reared in 20% Steinberg's solution, pH 6.5, containing antibiotics as indicated above.

3. The volumes of cytoplasm injected were estimated by calibrating the pipettes in the following way. Pipettes were filled to a mark with water, the water was then completely expelled with the pipette tip in air, and the diameter of the sphere formed at the pipette tip was measured with an ocular micrometer. The volumes delivered from the marked pipettes could then be calculated. Volumes of recipient eggs were similarly calculated from diameter measurements. The volumes of cytoplasm actually injected into eggs of *o/o* females ranged around 1–1.5% (56 injections), and 2.5–5% (211 injections) of the volumes of the recipient eggs. Recipient eggs ranged from 3.6 to 4.2 mm^3 in volume. Almost all recipients displayed a correction of the maternal effect of *o*. There was considerable variation in the degree of the correction, but in general the eggs injected with the larger volumes of normal cytoplasm were most improved, and in many cases approximated normal embryos in their development.

However, even in these embryos there is no sign of the formation of neural folds or other axial structures.[9]

An impressive aspect of these observations on untreated fertilized eggs of *o/o* females has to do with the consistency with which they express the maternal effect of gene *o*. Eggs of a given female are very uniform in their development, all stopping at the same stage and surviving for about the same period (2–3 days) before they cytolyze. Another point to be emphasized is that there is no indication that the maternal effect of *o* is influenced by the genotype established at fertilization. Thus, eggs of a given *o/o* female, fertilized by sperm from a heterozygous (+/*o*) male, all develop in the same fashion.[9]

Effects of Normal Egg Cytoplasm on the Development of Eggs of o/o Females.—Donor eggs from normal (+/+ or +/*o*) females were obtained by injecting the females with FSH and LH, and later collecting the eggs as the females spawned them (see Humphrey[12] for methods). Eggs collected in this way have completed the first meiosis and are in the metaphase of the second meiotic division at the time of spawning. The recipient eggs were collected following natural matings of *o/o* females with +/*o* or +/+ males. The eggs were removed from their capsules and were then set aside until either the time of second polar body formation or the time of first cleavage. If cytoplasm was transferred prior to the first cleavage, eggs in which normal second polar bodies were observed were chosen as recipients and placed in depressions in agar-bottomed dishes, alongside the donor eggs, in Steinberg's solution.[13] Cytoplasm transfers were performed by sucking animal hemisphere cytoplasm from a donor egg into a micropipette approximately 15–25 μ in diameter at the tip. When the desired amount of cytoplasm had been withdrawn, the pipette was removed from the donor egg, inserted without delay into the recipient egg, and the cytoplasm then slowly injected. A glass plug was placed in the hole in the vitelline membrane following removal of the pipette, to prevent leakage of cytoplasm (see Signoret, Briggs, and Humphrey[20]). For the alternative procedure, transfer of cytoplasm after first cleavage, eggs displaying normal furrows were chosen as recipients, and the same methods were used for cytoplasm transfer, except that injections were done into both blastomeres with a small (*ca.* 15 μ) pipette and glass plugs were not required. The two procedures gave similar results. In each the total volume of cytoplasm injected ranged from 1 to 5 per cent of the volume of the recipient eggs.

The development of the recipient eggs is summarized in Table 1, and provides a convincing demonstration of the fact that the maternal effect of gene *o* can be corrected by relatively small amounts of normal cytoplasm. Some of the recipient eggs failed to cleave normally and so could provide no test of the effect of the injected cytoplasm. However, the majority of the injected eggs cleaved in regular fashion, and formed blastulae of normal appearance. There were 186 such blastulae, 182 of which displayed clear-cut evidence of the corrective effect of the injected normal cytoplasm. These embryos varied somewhat, depending principally upon the amount of cytoplasm they had received. Small amounts (1% or less of the volume of the recipient eggs) led to an improvement in gastrulation, and usually to the formation of definite neural structures. Larger amounts (2–5%) led to much improved development, frequently approaching that of the normal control embryos. The best of these embryos conform to the description given below for embryos derived from *o/o* eggs injected with nuclear sap from ovarian oöcytes.

Relative Effects of Nuclear Sap and Cytoplasm of Normal Ovarian Eggs on the Development of Eggs of o/o Females.—The experiment described in the preceding part of this paper indicates that a product of the normal allele of gene *o* is present in the cytoplasm of mature eggs in a form capable of correcting the maternal effect of *o*. The experiment now to be described was done to determine the localization of the corrective material in ovarian eggs—prior to the time of breakdown of the large egg nucleus (germinal vesicle). Donor eggs for this experiment were obtained from fragments of ovaries of mature +/+ females which had been killed by decapitation. The ovary fragments were stored in a small amount of coelomic fluid in an airtight container at about 2°C. Eggs from such fragments were used over a period of 24–30 hr.

In order to obtain either nuclear sap or cytoplasm, the eggs were treated in the following way. Large eggs, ranging in diameter from 1.7 to 2.0 mm, were removed from the ovary fragment and placed singly in a hemispherical depression of a depression slide. Excess coelomic fluid was removed with small bits of filter paper. The eggs were then covered with Squibb mineral oil, and ripped open with the aid of two pairs of stainless steel watchmaker's forceps. Under mineral oil the cytoplasm remained fluid. As it flowed out of the opened egg, the large transparent germinal vesicle could usually be seen emerging from the animal hemisphere region. Once spotted, the germinal vesicle was moved to one side, away from the main mass of cytoplasm. Nuclear sap, appearing water-clear but rather viscous, was then sucked up into a fine (10-μ tip) micropipette and transferred into fertilized eggs of *o/o* females. Cytoplasm, taken from the main cytoplasmic mass, was similarly tested for its corrective effect on the development of eggs of *o/o* females.

The results of this experiment are given in Table 2, from which it appears that the nuclear sap of +/+ eggs contains in concentrated form the material capable of correcting the maternal effect of gene *o*. Quite small amounts of nuclear sap, in the range of 0.2–0.5 per cent of the volume of the recipient egg, led to normal or nearly normal development of many of the eggs of *o/o* females. The embryos listed as "normal" in Table 2 developed into postneurulae like those illustrated in Figure 1, many of which would be expected to attain normal larval stages. Some of these embryos developed infections and died, apparently for this reason. Others reached what appeared to be normal larval stages. They possessed a normal circulation, an apparently well-differentiated digestive system including a liver capable of secreting bile, and appeared to be actively swimming normal larvae. Such larvae

TABLE 2

EFFECTS OF NUCLEAR SAP AND CYTOPLASM FROM NORMAL OVARIAN OÖCYTES ON THE DEVELOPMENT OF EGGS OF *o/o* FEMALES

Material injected	Approx. volume–% of egg volume	No. of eggs injected	Blastulae		No correction of effect of *o* — arrested blast. and gast.	Correction of Effect of *o*			
			Partial	Complete		Gast.	Neur.	Postneurulae Abn.	"Normal"
Nothing (control)	0	0		196	196				
Normal oöcyte cytoplasm	1	35	1	32	18	6	10		
Normal oöcyte nuclear sap	0.2–0.5	43	5	33		3	3	17	14

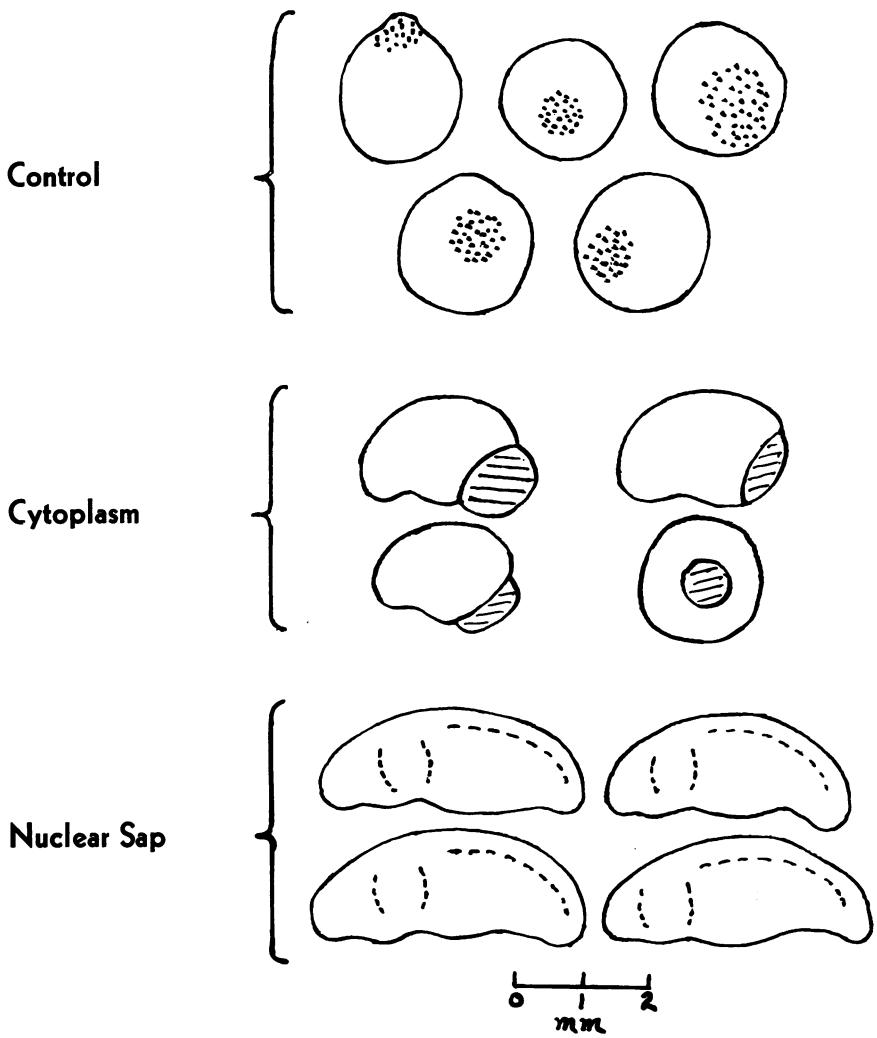

FIG. 1.—Camera lucida drawings illustrating the best development exhibited by *o/o* eggs following injections of normal ovarian oöcyte cytoplasm and nuclear sap (see Table 2). *Control:* Control *o/o* embryos were arrested at the early gastrula stage. In embryos cultured outside their capsules the pigmented hemisphere diminished gradually in size to the area indicated by the stippling. Embryos left in their capsules were arrested at the same stage, but did not show the diminution in the size of the pigmented hemisphere. *Oöcyte cytoplasm:* Eggs injected with oöcyte cytoplasm were arrested in the abnormal gastrula and neurula stages illustrated. The crosshatching indicates persistent yolk plugs. *Nuclear sap:* Eggs injected with nuclear sap were nearly normal and developed beyond the stage illustrated to advanced embryonic or early larval stages.

survived for a few weeks but failed to start feeding, became more and more wraith-like, and eventually died.

The cytoplasm of normal ovarian eggs exerts a detectable correction of the gene *o* effect, but it is clearly much weaker than the nuclear sap in this respect (see Table 2). Even though considerably larger volumes of cytoplasm were injected, the recipient eggs displayed marginal corrections, reaching abnormal neurula stages at best, as illustrated in Fig. 1.

Discussion.—As a result of the work so far completed, it is clear that gene *o*, when homozygous, brings about an important change in the egg cytoplasm during oögenesis, leading to a cessation of development much later on, at about the time when the main organ systems of the embryo are to be laid down. It is also clear that this gene effect is in the nature of a deficiency which can be corrected by some unknown component of the cytoplasm of normal mature eggs. Finally, the work described in this paper shows that this component, presumably a product of the normal allele of gene *o*, is retained in large amounts within the germinal vesicle of ovarian oöcytes. Several problems now present themselves concerning this potentially important gene product. For example, it would be highly desirable to know the locus of the gene controlling the product, the time when it is produced during oögenesis, whether it is produced in cell types other than eggs, and the mechanism of its action, especially in early embryonic development. However, all of these may perhaps be regarded as secondary to the problem of the chemical nature of the product or products controlled by the normal allele of *o*. Evidence already at hand indicates that it cannot pass through the plasma membrane,[11] suggesting that it may be a particulate or a large molecule. Also, its localization within the germinal vesicle shows that it cannot be identified with organelles or inclusions found only in the cytoplasm (e.g., yolk, mitochondria, Golgi apparatus, etc.). Some preliminary fractionation studies, currently being done in collaboration with J. T. Justus and R. R. Humphrey, indicate that the active material is a large molecule, but it is possible that particles of the size of ribosomes might also be present in the active fractions so far obtained. The simplest possibility is that the active material is the direct product of the normal allele of gene *o*, namely, RNA, and that this RNA or its products must be available to interact with the zygote genome in specific ways if development is to proceed beyond gastrulation. Of course, it would be most significant if this or other gene products, stored during oögenesis, could be related to the cytoplasmic localizations known from experimental embryology to control the pattern of early embryonic development. An interpretation of this type fits in with recent evidence showing that the RNA required for early amphibian development is produced and stored during oögenesis,[14–17] and with both recent and older evidence indicating that the zygote nuclei begin to function in essential and specific ways just before or at the time of gastrulation.[18, 19] Whether the gene product we have been discussing in this paper actually fits this interpretation remains to be seen.

Summary.—A recessive gene (*o*), recently discovered by Humphrey,[9] exerts a maternal effect in the axolotl, modifying the egg cytoplasm during oögenesis in such a way as to lead invariably to a cessation of development much later on, during gastrulation. The work reported in this paper shows that whole cytoplasm from mature normal (+/+ or +/*o*) eggs corrects the maternal effect of *o*. Fertilized eggs of *o*/*o* females, injected with normal cytoplasm at the one- or two-cell stage, are greatly improved in their development. It is also shown in the work reported here that the corrective component is present in much higher concentration in the nuclear sap than in the cytoplasm of ovarian eggs prior to maturation. These results indicate that the normal allele of gene *o* elaborates a product during oögenesis which accumulates initially in the nuclear sap. Later this product is dispersed in

the cytoplasm and subsequently plays an essential role in development beyond gastrulation.

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*SPECTRAL ANALYSIS OF THE BINDING OF ACRIDINE ORANGE TO POLYTENE CHROMOSOMES**

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In vitro studies on the binding of several acridine dyes, including acridine orange, to DNA have shown that binding occurs in two modes.[1-4] For low dye to nucleotide-pair ratios (about 1:4 or less), binding is in a mode in which acridine orange absorbs with a peak near 5000 Å and emits a short-lived fluorescence at about 5200 Å. For higher dye to nucleotide-pair ratios, a new mode of binding appears in which