EFFECTS OF HEAT AND STORAGE ON UNFERTILIZED AND FERTILIZED EGGS OF XENOPUS

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In African toads of the genus Xenopus, amplexus is inguinal and the eggs deposited by the female are immediately fertilized by the male. The fertilizability of the egg in water is of short duration so that the collection of unfertilized eggs laid by an isolated female and their subsequent insemination with a testicular macerate is usually without effect.

The eggs of Xenopus are normally deposited over several hours, and samples taken from the bottom of a laying tank are usually composed of many developmental stages.

These two characteristics of egg-laying in this toad genus have limited the otherwise general usefulness of the animals for developmental research.

The introduction of artificial fertilization methods for Xenopus have, in consequence, been welcome and in particular the techniques of Wolf & Hedrick (1971). The methodology of these authors have not only permitted the artificial fertilization of considerable numbers of eggs simultaneously (thereby permitting considerable synchrony in the early cleavage stages and, in turn, the opportunity to investigate cell cycle-dependent events) but also the extension of the period of fertilizability by storing the female gametes in high salt solutions.

Our laboratory has been exploiting and modifying the Wolf & Hedrick technique in order to extend to Xenopus several genetic routines available for other amphibia, and to undertake a program for the investigation of the effects of high temperature and overmaturity on the development of reproductive potential in treated embryos.

In their technique, Wolf & Hedrick drew attention to the artificial capacitation obtained when their testicular macerates in high salt were diluted. The fertilizability of such macerates is considerable, and most especially in the presence of eggs that have been stored in high salt in the unfertilized state. However, the concentration of sperm has to be carefully controlled if normal, diploid, development is to ensue; high concentrations of sperm engender considerable numbers of dispermic eggs, and thus induced triploidy is common. These same groups of fertilized eggs will also contain some eggs which develop as haploids, although the mode of origin of such individuals is at present obscure.

In 1947, Robert Briggs reported on the ability to obtain triploid individuals in Rana by subjecting diploid eggs to a heat shock shortly after the accomplishment of fertilization. These results were later extended to Xenopus by Smith (1958). The use of the Wolf & Hedrick technique

has allowed us to repeat Smith's experiments, but using large groups of synchronously fertilized eggs in which the relation of the time of treatment to the phases of the cell cycle was definable. Under these conditions, it has proven possible to establish the optimal treatment for the maximum yield of triploid embryos.

Since heat shock triploids are genetically different from the triploids obtained from dispermic eggs, the conjunction of high sperm count fertilizations with post-fertilization heat shocking has proven additive, so that not only large numbers of triploid, but also many tetraploid, larvae may be obtained.

The heat shocking of unfertilized eggs does not prejudice their chances for subsequent fertilization. The effects of such treatment are variable as far as the establishment of triploids is concerned, and they will be discussed at a later date.

Retention of unfertilized eggs in high salt does not prevent the normal expansion of the jelly coat investments of the egg. Nonetheless, it is possible to store the female gametes for up to 19 hours at 19°C and then to fertilize them by lowering the salt concentration and adding motile sperm. The eggs do not appear to be in a state of 'suspended animation' during this period (period of overmaturation) since their ability to be fertilized gradually declines, and they can be shown to be actively metabolizing protein. The length of the period of overmaturation also seems to bear some relation to the reproductive potential of the surviving larvae, as assessed by examining the number of germ cells resident in the gonads, and the most recent results of studies in this domain will be reported.

Briggs, R — The experimental production and development of triploid frog embryos. J.Exp.Zool. 106, 237-266 (1947)

Smith, S — Induction of triploidy in the South African clawed frog, Xenopus laevis (Daudin). Nature 181, 290 (1958)

Wolf,D & Hedrick,J — A molecular approach to fertilization. II Viability and artificial fertilization of Xenopus laevis gametes. Dev.Biol. 25, 348-359 (1971)

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Abstract - The Dual 5S Gene System in Xenopus

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At least two kinds of genes code for 5S ribosomal RNA in Xenopus. The dual 5S RNA gene system occurs in those species of animals which synthesize enormous amounts of ribosomes during oogenesis in their oocytes. This immense synthesis is supported by gene amplification of the 28S and 18S ribosomal RNA genes (rDNA), and an extra set of 5S RNA genes which only function during oogenesis in oocytes. In somatic cells of X.laevis a homogeneous 5S RNA is synthesized. In oocytes only about 10% of the 5S RNA is of the somatic type; about 60% has 6 nucleotides different from the somatic type. We refer to this as the principle oocyte-type 5S RNA. Other sequences make up the remainder of this RNA (minor oocyte-type 5S RNAs).

Control of the genes for these 5S RNAs seems to occur as follows: all types of 5S genes work in oocytes; oocyte-type genes are turned off in somatic cells.

The dual 5S RNA gene system has the following advantages for gene control studies. The transcription unit is known (5S RNA); it is small (120 nucleotides long); and, it has been completely sequenced. There is no precursor synthesized at the 5' end of the RNA. The initiation site for polymerase is known; it is the first nucleotide of the 5S RNA molecule. The polymerase form which transcribes 5S RNA in vivo has been identified as Form III. The oocyte-type 5S RNA has been purified from X.laevis and X.borealis (previously termed X.mulleri in our publications). Restriction enzyme maps have been constructed of these DNAs, regions of each repeat have been sequenced, and both DNAs have been cloned in E. coli by recombinant DNA methodology. One other 5S DNA component has been purified from the genome of X.laevis and X.borealis. The minor X.laevis 5S DNA component has been sequenced in part. It is not the somatic 5S DNA. The minor X.borealis 5S DNA component is being investigated at present.

Our goal in these experiments is to reconstruct faithful synthesis of 5S RNA in vitro as well as the dual 5S gene control system in vitro using purified oocyte-type and somatic-type 5S DNAs.

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Title

Structural gene sets active during sea urchin oogenesis and embryogenesis, and the persistence of maternal mRNA

Abstract

Measurements of embryo mRNA complexity made by the RNA excess hybridization method are reviewed. Most of the sequence complexity of embryo mRNA populations is included in about 10% of the mRNA, termed the "complex" class of the mRNA. Complex class mRNAs are present at one or a few copies per cell, which prevalent mRNAs exist in at least 50 copies per cell. Analyses of data on mammalian liver enzymes and mRNA populations demonstrate complex and prevalent mRNA classes in this tissue as well, and show that some of the rare mRNAs are responsible for the maintenance of histospecific liver enzymes. The complex class mRNA of sea urchin embryos occurs in stage-specific sets. By preparing a single copy DNA tracer complementary to oocyte RNA, the appearance during oogenesis and the disappearance during embryogenesis of the maternal sequence set are measured. Most complex class embryo mRNA sequences are represented in oocyte RNA, but the fraction of oocyte RNA sequences persisting in the embryo decreases as development proceeds. Maternal mRNA sequences disappear from polysomes prior to their disappearance from the total embryo cytoplasm. Synthesis and turnover kinetics of complex and prevalent mRNA populations are described, and calculations of minimum per gene transcription rates presented.

The Structure of Ribosomal DNA

Igor B. Dawid

Ribosomal DNA is the DNA component which codes for the precursor to the two large RNAs of the cytoplasmic ribosome. In all organisms the ribosomal DNA is a repeated component. Ribosomal DNA from *Xenopus laevis* was the first animal gene to be isolated in pure form. This DNA component has been studied in great detail. *Xenopus* ribosomal DNA is located in one region, the nucleolus organizer region, of one of the 18 haploid chromosomes. About 500 copies of a basic repeating unit are concentrated in this region. Each repeating unit consists of a region which codes for the 40S RNA precursor molecule and a nontranscribed spacer region. The length of the spacer differs from one repeat to the next even within a single nucleolus organizer. A large segment within the nontranscribed spacer is composed of small subrepeats. Long spacers are distinguished from short spacers by having more of these subrepeats. Along the DNA strand of the nucleolar organizer region a DNA repeats with short and long spacers alternate in a highly scrambled pattern.

To obtain a more general view of the structure of ribosomal DNA in animals we have studied this component in *Drosophila melanogaster*. This animal is particularly interesting because of the extensive information available on the genetics of its ribosomal locus. In *Drosophila* one nucleolar organizer region is located on

the X chromosome and one on the Y chromosome. In the wild type about 200 copies of the ribosomal repeating unit are clustered at each nucleolus organizer.

We have purified Drosophila rDNA and analyzed its structure. There are two basic types of repeating units. One type with a length of about 11 kb consists of a gene for the 38S ribosomal RNA precursor and a nontranscribed spacer. This type of repeating unit resembles the structure of the Xenopus rDNA unit. The second type of Drosophila repeating unit contains an insertion of a DNA sequence within the gene for 28S rRNA. This insertion of DNA sequences which do not code for 28S rRNA means that the 28S gene is split into two portions. We do not know whether this type of rDNA is transcribed into RNA in the fly. The size of this insertion is variable and occurs in multiples of 500 bases. The major component of inserted repeats has a 5 kb insertion within the 28S gene.

By studying the pattern produced from ribosomal DNA with restriction enzymes we find that the X chromosomal nucleolar organizer contains about 1/3 of repeating units without insertions in 2/3 of units with insertions. In contrast, rDNA on the Y chromosome does not contain any repeats with long insertions and may contain no insertions at all.

The nontranscribed spacer region which separates the end of the 28S gene from the beginning of the next transcription unit is also variable in length. However, this length variability is relatively

slight and most nontranscribed spacers are similar to each other. We have analyzed the arrangement of repeating units with and without insertions in the 28S gene along the DNA strand in the X chromosome nucleolus organizer. The result is that repeats with and without insertion are interspersed in a pattern that is close to a random scrambling.

These observations on length heterogeneity of ribosomal DNA repeats in Xenopus and Drosophila may be interpreted in terms of models for the evolution of tandomly repeated genes. Different models will be considered in this context.

(Workshop presentation)

Gene Regulation in Transferred Somatic Cell Nuclei

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A central issue in developmental genetics is the mechanism by which the cytoplasm effects nuclear expression.

This problem may be approached by transferring nuclei of differentiated cell types which normally synthesize a specific pattern of proteins into oocytes which are synthesizing a different set of gene products. Recently, Gurdon et. al.(1976) transferred HeLa cell nuclei into Xenopus laevis oocytes and examined patterns of newly synthesized proteins by 2 dimensional gel electrophoresis. Results of these experiments have shown that HeLa specific proteins are being synthesized by the transferred nuclei. More importantly however, there appears to be selectivity in that only a few of the recognizable HeLa gene products are expressed in the oocyte.

In the present investigation attempts have been made to analyze the effect of oocyte cytoplasm on the expression of specific genes of differentiated cell nuclei, utilizing an interspecific hybrid combination of Ambystoma texanum, and Ambystoma mexicanum(axolotl). The tissue specific enzyme alcohol dehydrogenase(ADH), found mainly in the liver, and lactate dehydrogenase(LDH) found in liver and oocytes are used as markers of gene activity. Both ADH and LDH show species specific patterns on starch gels which permits detection of enzymes synthesized by texanum liver nuclei following their transfer into axolotl oocytes. Analysis of recipient oocytes 1-3 weeks after transfer reveals the presence of newly synthesized texanum LDH, but not ADH. Appropriate controls were carried out to eliminate the possibility that these products were the result of transferred texanum mRNA.

These results indicate that in the case of ADH and LDH the oocyte cytoplasm appears to be able to regulate the synthetic activity of transferred somatic cell nuclei so as to conform to the oocytes normal synthetic output.

Gurdon, J.B., DeRobertis, E.M., and Partington, G.(1976) Nature, 260, 116.

The Developmental Significance of Lampbrush Chromosomes

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The title of this abstract is intended to focus attention on the fact that we know very little about the developmental significance of lampbrush chromosomes. Despite extensive study and equally extensive speculation, agreement is possible on only a few general statements. Nearly everyone believes that the lampbrush condition represents a transcriptionally active state of the chromosomes. The paired lateral loops are the active regions, consisting of a DNA axis from which extend closely spaced RNA transcripts. Electron microscopy shows the transcripts to be extraordinarily long (many μm), but their biochemical characterization has only begun. Many basic questions remain incompletely answered. In what sense is a loop a transcriptional unit? Why are there thousands of active loop pairs throughout oogenesis? In what sense do the chromomeres and loop pairs of amphibian oocytes correspond to the bands of dipteran polytene chromosomes? What fraction of the genome is transcribed during oogenesis? What fraction of the lampbrush RNA is utilized for translation during embryonic stages? Possible approaches to these questions will be discussed, with emphasis on probes for analyzing the RNA transcripts of the loops.

Abstract for Meeting in Bloomington, Indiana,

October, 1976.

The injection of nuclei and DNA into Xenopus oocytes.

by J.B. Gurdon, E.M. De Robertis, G.A. Partington, and J.E. Mertz. MRC Laboratory of Molecular Biology, Hills Road, Cambridge, UK.

The experiments to be described are designed to elucidate mechanisms of gene control and especially those which operate in oogenesis.

Methods have been developed by which multiple nuclei can be harmlessly transplanted into the cytoplasm, the germinal vesicle, or the dispersed GV contents of oocytes. Injected nuclei enlarge greatly, exchange proteins with the oocyte cytoplasm, and synthesize RNA continuously for as long as the oocytes remain healthy. Injected HeLa cell nuclei synthesize RNA which codes for some of the proteins synthesized by cultured HeLa cells. These proteins have been recognized by 2-dimensional protein analysis. Several independent kinds of evidence show that the HeLa proteins synthesized in oocytes depend on transcription from the injected HeLa cells, and are not attributable to carried over messenger RNA or nuclear RNA. Gene expression by HeLa nuclei in oocytes is selective, in so far as only a minority (3/20) of the recognizable HeLa proteins are synthesized in oocytes.

We have used these methods to ask whether genes which are inactive in somatic cells become activated when the nuclei in which they are contained are transplanted to oocytes. The experiments involve the transfer of nuclei from cultured Xenopus kidney cells to oocytes of Pleurodeles, and the recognition of gene expression by 2-dimensional protein analysis. In a preliminary report of these experiments (De Robertis, Gurdon, Partington, Mertz, and Laskey: "Biochemistry of the Cell Nucleus", Biochem. Socy. Symp., July 1976) evidence for the induced activation of oocyte-expressed (but kidney unexpressed) genes has been obtained. It therefore appears that oocytes may contain substances or conditions which can induce new gene activity, apart from permitting the continued expression of previously active genes.

In an attempt to investigate the molecular mechanism of these effects, Mertz and Gurdon have injected various kinds of purified DNAs into oocytes, and recognized transcription by appropriate RNA:DNA hybridization experiments. These experiments represent a continuation of experiments reported by Gurdon and Brown (Symposium on "The molecular biology of the mammalian genetic apparatus - its relationship to cancer, aging, and medical genetics" December, 1975) in which transcripts from purified ribosomal or 5S genes were found in embryos after the injection of purified ribosomal or 5S DNA into fertilized eggs. The present state of DNA injection experiments will be reported.

The Germ Plasm of *Drosophila*: An Experimental System for the Analysis of Determination

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The posterior polar plasm of *Drosophila melanogaster* may provide a model for understanding processes involved in cellular determination during early embryonic development. The presence of germ cell determinants in the posterior tip of the egg has been demonstrated by transplanting this material to new locations and showing that cells, induced at the site of transplantation, can function as germ cells. Furthermore, by utilizing similar procedures, the posterior polar plasm of late stage oocytes has been shown to be functional in inducing germ cells, thus demonstrating that the active components are formed during oogenesis. A number of maternal effect mutations affecting germ cell formation have been analyzed ultrastructurally and the mutant phenotype described. Ultrastructural studies have focused on polar granules, the unique organelle of the polar plasm, and these studies indicate that they are nonmembrane bound structures containing RNA and protein. Although ultrastructural observations have suggested that the protein components of polar granules, may be continuous in the cytoplasm of germ cells during the whole life cycle, nucleo-cytoplasmic hybrid pole cells indicate that the structure of the polar granule is dependent upon the nucleus in each new generation of oocytes. Finally, attempts are being made to obtain a purified polar granule fraction in order to test their role in germ cell determination. Initial results indicate that clusters of ribosomes are attached to polar granules. Pole cells have recently been isolated as an enriched source of polar granules. These isolated cells will also provide an opportunity to analyze the unique traits of these cells at the time that they become segregated from the remaining cells of the embryo.

Analysis of the neural induction system of the amphibian embryo with ultraviolet irradiation

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Ultraviolet irradiation of the vegetal hemisphere of the fertilized amphibian egg prior to first cleavage results in the embryo developing an incomplete set of neural structures. Irradiated eggs from various species of amphibia have been tested and they all display a characteristic set of defects in neural morphogenesis, including a shortened axial length and microcephaly. Mapping experiments have demonstrated that the future dorsal side of the egg displays the highest sensitivity to irradiation. Irradiation of eggs with U.V. of various wavelengths was performed to determine the action spectrum of the target. Preliminary results of those experiments will be discussed.

The effects of irradiation on the various morphogenetic processes which contribute to the embryo's capacity to complete neural development including cleavage division, formation of the dorsal lip, invagination at gastrulation, and neural induction by the primary organizer were examined. Various cell marking techniques, histological analyses, and grafting experiments were employed for those studies. The results indicate that U.V. can affect each of the above mentioned processes. A decrease in the capacity for invagination during gastrulation and a dimunition in the inducing capacity of the primary organizer can, however, account for defective neurulation in irradiated embryos.

Those analyses have permitted the reduction of the problem of the induction of the primary embryonic axis to the analysis of its component steps. A correction of the defects in neural development can be achieved in several ways, the most effective being embryological grafting techniques. Replacement of either the dorsal lip or the primary organizer of an irradiated embryo with the analogous part from a non-irradiated embryo promotes substantial neural development.

(Workshop presentation)

nc: A Microtubule Assembly Mutant in the Mexican Axolotl

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Eggs spawned by nc female axolotls appear to be morphologically normal. They show normal interaction with sperm, which enter and cause activation of the eggs. However, no mitotic apparatus or cytasters are assembled and no cleavage occurs.

We have found that the lesion in the nc mutant eggs can be partially corrected by injection of microtubule fragments or basal bodies. The best correction was achieved by injection of outer-nine doublet tubules prepared from sea urchin sperm tail axonemes. No cleavage results from injection of soluble tubulin, buffer, non-tubulin particles or normal germinal vesicle nucleoplasm. Cleavage is colchicine-sensitive, and so involves microtubule assemble. Electron microscope observations of corrected nc eggs show microtubule arrays similar to those of the normal mitotic spindle.

The tubulin pool size of nc eggs was found to be normal by ^{3}H-colchicine binding, and isolated nc egg tubulin appears to be identical to normal egg tubulin.

We suggest that nc eggs are defective in a step leading to the formation of microtubule nucleating centers.

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Synthesis of Stable and Unstable RNA in Xenopus laevis Oocytes

It is clear that the major product of transcription in somatic cells is heterogeneous nuclear RNA (hnRNA), characterized generally by its rapid turnover and limited transport to the cytoplasm, as well as by its larger size and greater complexity relative to polysomal message. In contrast, studies on RNA metabolism in oocytes have emphasized almost exclusively the synthesis and accumulation of stable RNA's, including complex heterogeneous RNA's. However, earlier studies from our lab on RNA synthesis in Xenopus stage 6 oocytes (growth complete) showed that the kinetics of incorporation of ^{3}H-GTP into RNA are biphasic, suggesting the synthesis of both stable and unstable RNA's. In this report, we describe experiments in which the newly synthesized RNA in stage 6 oocytes has been characterized additionally by base composition and gel electrophoresis. Based on these studies, the kinetics of synthesis and accumulation of ribosomal and nonribosomal (heterogeneous) RNA's have been followed. From the kinetics of total RNA synthesis, we estimate that only about 28% of the synthetic activity is devoted to stable species (over a 24 hour period) which accumulate. About 90% of this stable accumulation is devoted to ribosomal RNA. Heterogeneous RNA's account for 58% of the initial synthetic activity, and appear to contain at least 3 kinetic components, one (about 50%) with a half life of approximately 30 min, a second (45%) with a minimum half life of about 4 hours, and a minor component (5% of total heterogeneous RNA) which is stable over the course of our experiments. Based on the nuclear cytoplasmic distribution of the heterogeneous RNA's, the short half life component appears to be equivalent, by virtue of its size, base composition, rapid turnover and location, to the hnRNA observed in somatic cells. The heterogeneous

RNA with a half life of 4 hours, in contrast to somatic cells, also appears to be restricted to the nucleus. Finally, the stable component appears to be localized, after a lag, exclusively within the cytoplasm. Preliminary studies show that RNA containing poly (A) also is synthesized with linear kinetics, at least over a 6 hour period. Part of this may be included within the stable heterogeneous component. Thus, like somatic cells, only a small portion of the heterogeneous RNA synthesized in stage 6 oocytes ends up in the cytoplasm, presumably as potential mRNA, while the bulk turns over within the nucleus.

It is well known that stage 6 oocytes contain a "stockpile" of maternal mRNA which, including repetitive and unique transcripts, may total 180-260 ng. There are several reports which indicate that the final content of maternal mRNA may be attained by the maximal lampbrush stage or earlier. On the other hand, the rate of accumulation of the stable heterogeneous component, is such that, assuming no turnover, oocytes could accumulate about 2 ng per day even at stage 6. They could essentially double the amount present at stage 3, assuming a constant rate and about 3 months duration between stage 3 and stage 6. Hence, it is suggested that even the "stable" heterogeneous RNA turns over with a defined half life, albeit longer than a day (the length of our experiments). Dependent on the actual half life, two interpretations of the data are possible. First, maternal mRNA is synthesized and accumulated during or prior to stage 3, while heterogeneous RNA synthesized later would represent a subset of the message population, perhaps only a few percent, including mRNA needed for translation during oogenesis. Alternatively, maternal mRNA could turn over from the beginning, with the final quantity representing a steady state level. In this case, synthesis would go on continuously at more or less similar rates, regardless of whether or not lampbrush chromosomes were present.