All components required for the eventual activation of muscle-specific actin genes are localized in the subequatorial region of an uncleaved amphibian egg

(activin gene transcription/cytoplasmic localization)

J. B. Gurdon, T. J. Mohun, Sharon Fairman, and S. Brennan

CRC Molecular Embryology Unit, Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, England

Contributed by J. B. Gurdon, September 4, 1984

ABSTRACT Fertilized Xenopus eggs have been ligated with a hair loop into separate fragments before the first cleavage. The plane of the ligation was varied in relation to the animal-vegetal and dorso-ventral axes. The fragments that contained a nucleus were cultured for 24 hr until controls reached the neurula stage; they were then analyzed by S1 nuclease protection for their content of muscle-specific actin mRNA, using a gene-specific probe. We find that all egg components required for the eventual activation of these actin genes are localized, already at the 1-cell stage, in a region below the equator, and mostly on the dorsal (grey crescent) side. This material subsequently occupies the equivalent position in 8-cell and 32-cell embryos. We interpret our results, in combination with the previous work of others, to mean that mesoderm (including muscle) formation in Amphibia depends both on cytoplasmic substances already localized in the egg as well as on inductive cell interactions during cleavage.

An important question in embryology is whether a fertilized but uncleaved egg contains substances already localized in a way that is related to future cell differentiation, or whether such substances first appear and become localized at a later multicellular stage. The question is significant because the localization of materials in a single cell, the egg, is most likely to depend on large molecules or complexes, whereas cell interactions may well involve small molecules capable of passing rapidly from cell to cell.

There are several examples in invertebrate animals in which one region of egg cytoplasm seems to be related to a particular type of differentiation. The most celebrated cases are insect pole plasm (1) (related to egg and sperm formation) and the yellow cytoplasm in ascidian eggs (related to tail muscle formation) (reviewed in refs. 2 and 3). A more common situation is that in which a particular region of egg cytoplasm becomes localized within a certain cell lineage by asymmetric early cleavage divisions, as in the photocyte and cilia-forming cells of Ctenophores (4). In the vertebrates, the only definite example of either kind is the germ plasm of Amphibia (5), which appears to be functionally equivalent to the pole plasm of insects. Here we describe ligature experiments in Xenopus and the use of a gene-specific probe to investigate the localization of materials required for the activation of muscle-specific a-actin genes. Rather surprisingly, we find that these materials are already localized in a particular region of the uncleaved egg.

MATERIALS AND METHODS

Eggs and Embryos. Eggs laid after hormone injection were artificially fertilized as described (6). Developmental stages are those of Nieuwkoop and Faber (7).

Hair-Loop Ligations. Eggs were dejelled in saline containing 2% cysteine (pH 8.0), washed, and the vitelline membrane was removed manually with forceps. Eggs were ligated with a thin Louise hair-loop in the desired orientation in modified Barth saline (MBS) (8), between 30 and 60 min after fertilization (30%-60% of the length of the first cell cycle). When the constriction was completed (see Results), egg fragments were cultured in MBS at ~20°C.

Blastomere Separations. Eight-cell embryos, after removal of the jelly and vitelline membrane, were transferred to Ca2+/Mg2+-free MBS, while blastomeres were separated. The animal or vegetal half-embryos were then cultured in MBS. The vegetal tier of cells was removed from 32-cell embryos by cutting through the cells about 3/4 of the way down from the animal pole. The rapidly leaking vegetal cells were gradually cleaned away over the next hour, leaving only the more animal cells as part of the remaining embryo. By this means, all cells that reached the vegetal pole were removed.

S1 Nuclease Assays. The amount of a-actin mRNA was determined in each embryo or part embryo, individually, as described (9, 10). The probe used (M2) recognizes cardiac a-actin RNA (see Fig. 2), which is a major product of axial muscle in Xenopus embryos.

RESULTS

One-Cell Ligations. If the components required for a-actin gene activation are already localized at the one-cell stage, it should be possible to obtain some fragments of an egg that do, and others that do not, show a-actin gene transcription. If, conversely, the required substances are evenly spread throughout an egg (or do not exist at this stage), all egg fragments should activate (or fail to activate) muscle-specific genes. Eggs were taken for constriction 30-45 min after fertilization, when the first cytoplasmic cleavage takes place at ~100 min. At first the constriction was tightened to between 1/2 and 3/4 of the full extent. After ~15 min, the constriction was completed. All eggs were therefore divided into two entirely separate fragments well before the time of the first cleavage. When contractions were to be oriented in relation to the sperm entry point, only eggs in which this could be clearly seen as a black spot above the equator (i.e., within the pigmented hemisphere) were used. After ligation, embryo fragments were cultured for about 24 hr, until control whole embryos (not ligated) had reached stages 18-20.

The ligated egg fragments that happen to contain the egg nucleus (near the animal pole) or the sperm nucleus (near the dark spot marking the sperm entry point), or both, divide in synchrony with unligated controls. In the absence of a vitelline membrane, blastomeres tend to flatten in the plane of the culture dish. Consequently, the mitotic spindle, which is characteristically oriented in the longest cell dimension (11), causes embryos to form the flattened structures seen in Fig. 1 A-D. Some of the types of development seen after 24 hr, when controls are at stage 18, are shown in Fig. 1 E-I.
Fig. 1. Development of *Xenopus* egg fragments after ligation at the one-cell stage. (A–D) Two, 4, 8, and 32/64-cell stages of an animal hemisphere fragment. Hair loop and uncleaved vegetal hemisphere can be seen in A–C. (E–H) Embryos 24 hr after fertilization, when controls had reached stage 18/20. (E) From an animal hemisphere. (F) From sperm entry point (ventral) 1/3. (G) From sperm entry point one-half. (H) From grey crescent (dorsal) half. (I) Non-nucleate vegetal hemisphere cytoplasm 24 hr after isolation. Embryos in F, G, and H synthesized α-actin RNA; those in E and I did not.

Fragments derived from 1/2 or less of an egg centered around the animal pole always form flat discs, with no indication of an axis (Fig. 1E). In contrast, those egg fragments that include part of the vegetal hemisphere usually form embryos with more or less evident axial structures (Fig. 1F and G); those fragments that contain dorso-vegetal cytoplasm (see Fig. 3A) always have very obvious axial structures (Fig. 1H). Egg fragments that contain no nucleus, such as the vegetal 1/3, at first fail to divide, but eventually may fragment into variably sized pieces of cytoplasm that contain no nuclei (Fig. 1I). We conclude, on morphological criteria, that components required for axis formation are already localized at the one-cell stage.

**Actin Gene Transcription.** The transcription of muscle-specific actin genes in cultured egg fragments has been assessed, as in our previous actin gene work (referred to below), by use of a gene-specific probe. Cardiac α-actin is one of the main contractile proteins synthesized by muscle cells of *Xenopus* embryos, and it is not detectable in the future heart region at the neurula stage. For the experiments reported here, we have used a 330-nucleotide length of DNA complementary to part of the 3′ untranslated region, and to a small part of the coding region, of *Xenopus laevis* cardiac α-actin mRNA, as indicated in Fig. 2C. The mRNA for cardiac actin usually protects 250 bases of this probe from S1 nuclease digestion, whereas no part of this probe approaching this size is protected by the mRNAs for other actins or for any other genes (9, 10). The use of this probe is illustrated in Fig. 2. The presence of cardiac actin transcripts is indicated by a strong band ~250 nucleotides long (Fig. 2B, stage 19). In the absence of such transcripts (Fig. 2B, stage 9), no such band is seen.

Embryo fragments were reared from ligated eggs until whole embryo controls had reached stage 18–20, and their RNA was then analyzed as described above. Typical results are shown in Fig. 2A. The 1/3-most animal part of an egg never synthesizes cardiac actin RNA, whereas the 2/3-most animal part usually does so and the dorso-vegetal (grey crescent) region opposite the sperm entry point always does so. The results of 98 analyses are summarized in Fig. 3. The main conclusion is that materials in the animal 1/3 of an uncleaved egg are not required for cardiac actin gene transcription. The region that is required is in the vegetal hemisphere and is more concentrated on the side of the egg opposite the sperm entry point, although clearly present to some extent on the side of the sperm entry point. Fig. 3 summarizes these experiments and indicates, on the basis of our ligation experiments, the most likely location of substances required for activating muscle-specific actin genes.

Our claim for localization depends critically on our result that the animal half of an egg does not activate α-actin genes. The simplest explanation for this would be that these fragments are unhealthy. However, we know that this is not so, because when cultured for a second day until whole embryo controls have reached stage 26, these animal halves develop cilia and rotate in the culture dish. The formation of cilia is a characteristic ectodermal differentiation (12), and we have not seen cilia in equatorial or vegetal regions of embryos when they have reached the same stage. We have also tested the possibility that animal region fragments are too small for cardiac actin expression by combining from two to four animal fragments at the early blastula stage. Such aggregates are as large as a whole embryo (and contain more cells) at the same stage, but they have synthesized no cardiac actin transcripts by stage 20 (Fig. 2A, animal 1/3 × 2).

In summary, a ligated animal 1/3 of an uncleaved egg will not transcribe cardiac actin genes but will form cilia. Conversely, any egg fragment that contains only vegetal hemi-
sphere cytoplasm, especially from the dorsal side, synthesizes cardiac actin RNA at about the normal stage in development but does not form cilia. Therefore, substances required for α-actin gene transcription are localized at the one-cell stage.

Separation of Early Blastomeres. The fact that all components required for the eventual activation of cardiac actin genes are localized in one region of an uncleaved egg does not exclude an essential role for cell interactions. Nieuwkoop and colleagues (13) have clearly established that vegetal cells of a blastula can induce animal region cells to become mesodermal and to form muscle. The subequatorial localization defined by our one-cell ligation experiments can indeed be interpreted, in accord with this concept, as two regions of egg cytoplasm that are included in different cells during cleavage and that must interact with each other, perhaps by induction, for mesoderm formation to take place. The other interpretation of our results is that the subequatorial cytoplasm in an egg is directly related to subsequent ac-

---

**FIG. 2.** S1 nuclease assays of α-actin RNA. Lanes show analysis of RNA from an embryo or part of an embryo as illustrated in Figs. 3 and 4. Total RNA (2–4 μg) (between one-half and whole embryo) was used in each analysis. Presence of cardiac actin mRNA is indicated by a band of label ~250 nucleotides long (arrowhead), which is sometimes resolved into a doublet. High molecular weight label toward top of some lanes varies in amount from one batch of analyses to another and is unrelated to the amount of cardiac actin RNA. **(A)** Analysis of embryos from ligated one-cell stages. **(B)** Analyses of embryos from divided 8-cell and 32-cell stages. Abbreviations are as in Figs. 3 and 4. **(C)** Diagram of the prime-cut probe used for these analyses (for details, see ref. 9). The full-length probe (380 nucleotides) has some M13 sequence at each end. The remaining 330 nucleotides are able to hybridize with cardiac actin mRNA. However, this type of mRNA is terminated at two positions, just after the two polyadenylation sites (a). The predominant cardiac actin mRNA of normal embryos terminates at the first of these and protects 250 nucleotides of the labeled probe.

---

**FIG. 3.** Summary of egg ligation experiments. **(A)** Axes of a fertilized Xenopus egg. **(B)** Arrows point to the fragment of an uncleaved egg that was grown and subsequently analyzed. In some cases, animal 1/3 and vegetal 2/3 fragments were derived from the same egg. Third and fourth columns summarize the results of S1 nuclease analyses of RNA. Last column gives an overall assessment of the strength of the cardiac actin gene activating capacity of the egg fragments tested, on the basis of S1 nuclease assays. +, Strong activation in all cases; ±, weak activation in some cases, none in others; −, no activation in nearly all cases. **(C)** Diagrammatic interpretation of the results; solid spots indicate the presumed location and concentration of the cytoplasmic substances required to eventually activate cardiac actin genes.
tin gene transcription, without any need for cell interactions. The merits of these two concepts can be usefully compared by the following blastomere separation and deletion experiments.

The earliest time in *Xenopus* development at which an inductive interaction between vegetal and animal region cells could take place is at the eight-cell stage, when a horizontal cleavage separates vegetal from animal blastomeres. As indicated in Figs. 2B and 4A and B, the separated four vegetal blastomeres of an eight-cell embryo synthesize cardiac actin RNA when cultured for a day, to about the same extent as a whole embryo. The four animal blastomeres never synthesize cardiac actin RNA, unless, as happens not infrequently, the dorsal two of these (opposite the sperm entry point) include vegetal hemisphere cytoplasm (see Fig. 4B). There is considerable variation between eggs in the position of cleavage furrows with respect to the animal and vegetal poles, and this might explain why Kageura and Yamana (14, 15) sometimes observe muscle differentiation in four isolated animal cells from an eight-cell embryo. In all other respects, our eight-cell results agree with theirs. It therefore appears that the subequatorial cytoplasm in a fertilized egg occupies the equivalent location in an eight-cell embryo; that is, it is entirely within the four vegetal blastomeres of a horizontally cleaved eight-cell embryo. We conclude that, if cell interactions are required for mesoderm formation, these do not take place to a significant extent at the eight-cell stage.

The next stage at which a new layer of cells is formed within the subequatorial zone is at the 32-cell stage, because when forming the 16-cell stage, the vegetal blastomeres of the 8-cell embryo divide vertically (Fig. 4C). We have removed all blastomeres of a recently formed 32-cell stage embryo that are at or near the vegetal pole. We find that cardiac actin transcripts are formed (Figs. 2B and 4D), a result in agreement with a morphological analysis of Nakamura and Takasaki (16), although the amount synthesized is significantly reduced in some cases. Therefore, those blastomeres in the subequatorial zone, which are believed to be direct ancestors to much of the embryonic axial muscle (see Discussion), appear not to require an induction from the underlying vegetal tier of cells. We conclude that, at least up to the 32-cell stage, actin gene activation does not depend on an inductive interaction involving cells that touch the vegetal pole.

**DISCUSSION**

The main conclusion from these experiments is that all cytoplasmic components of an egg that are necessary for the eventual transcription of muscle-specific actin genes are localized in a subequatorial region of the uncleaved *Xenopus* egg. By a similar argument, the components required for cilia formation are localized in the animal hemisphere. The only previous example of a specific cytoplasmic component localized in amphibian eggs is the germ plasm. It has been known for some time that the dorso-vegetal surface of amphibian eggs is especially sensitive to ultraviolet irradiation (17, 18), but it now seems likely that this treatment inhibits a general cytoplasmic rearrangement that normally follows fertilization and not that it destroys a specific cytoplasmic substance (19, 20).

Within the invertebrates, it has been possible, in a few species, to cut uncleaved eggs so that some of the resulting fragments survive long enough to assess their differentiation by morphological criteria. Such experiments are informative in the present context only if the orientation of the cut is known in relation to at least one axis of the egg. This appears to have been first achieved by Driesch and Morgan (21), who found that division of the fertilized but uncleaved egg of the Ctenophore *Beroe* resulted in small embryos, each containing the proportionately reduced number of comb plates, a result confirmed and extended by Fischel (22). The cytoplasmic materials required for the differentiation of the eight comb plates (containing cilia) are therefore fixed in amount and presumably localized in the uncleaved fertilized egg. A similar conclusion can be drawn from experiments on fertilized but uncleaved eggs of *Cerebratulus*, a nemertine worm, by Yatsu (23) and subsequently by Freeman (24). In this case, there is a differential localization of materials required for the formation of the apical organ, compared to those needed for the ciliated lobes and gut. Lastly, Delage (25), and in a more extensive experimental series Wilson (26), removed the polar lobe of fertilized but uncleaved eggs of the mollusc *Dentalium* and showed that this region of cytoplasm alone contains materials essential for the formation of the apical organ and of post-trochal structures such as the shell, shell-gland, and foot. From these and other examples (27, 28), it seems that cytoplasmic substances required for regional differentiation are commonly localized in the fertilized but uncleaved eggs of invertebrates. Our results with *Xenopus* suggest that cytoplasmic localizations may have a

---

**Fig. 4.** Diagrammatic summary of animal–vegetal region separations in early *Xenopus* embryos. Orientation of each embryo is the same as in Fig. 3—i.e., sperm entry point to the right, grey crescent region (dorsal) to the left, and animal pole uppermost. Embryos are therefore viewed from the right-hand side, so that only 4 cells of an 8-cell embryo can be seen. The cytoplasmic cleavage converting a 4-cell to 8-cell embryo is often diagonal on the dorsal side (star), such that animal 4/8 fragments contain some equatorial cytoplasm and may subsequently transcribe cardiac actin genes to a low level. Right-hand column summarizes the results of S1 nuclease assays (at least 4 per row). +, Strong cardiac actin signal in each case; \(\pm\), weak signal in most cases; –, no detectable signal in each case.
similar role in early amphibian development to that generally
accepted for invertebrate eggs.

Are cell interactions required for α-actin gene activation?
The most direct interpretation of our results, we believe, is
that the subequatorial cytoplasm of an egg permits embryon-
ic cells containing it to transcribe α-actin genes at a later
stage. Another possibility is that this region of an egg con-
tains material that enables cells receiving it to induce other
cells to activate their α-actin genes. We have argued that an
induction does not take place at any time up to and including
the 32-cell stage. It might take place between any two cell
layers subsequently formed from the third tier of a 32-cell
embryo; however, this would require either a surprisingly
precise localization of substances within the subequatorial
layer of an egg or a segregation of materials among daughters
of the 32-cell third tier and a subsequent inductive interac-
tion among these. A third possibility is that muscle formation
depends on "positional values," which would move in com-
ensation when cells are removed from 8-cell or 32-cell em-
bryos. A distinction between these possibilities would be
helped by a knowledge of the lineage of axial muscle cells in
intact embryos. Unfortunately this is not yet certain for Xen-
opus; Nakamura and Kishiyama (29), Takasaki and Yagura
(30), and Nakamura et al. (31), using a vital dye all agree that
the third tier of a 32-cell embryo is the major contributor to
muscle, with a minor and variable contribution from second
tier cells. On the other hand, J. Cooke (personal communi-
cation), using horseradish-peroxidase injection, sees a major
contribution from the second tier.

The simplest way of assimilating the results we report here
with those previously contributed by others seems to us the
following. We propose that the subequatorial cytoplasm of a
fertilized egg directly commits those blastula cells into which
this cytoplasm passes to become mesodermal (and to have
the capacity to activate their cardiac actin genes), without
the need for any cell interactions. These may be referred to
as "primary" mesoderm cells. We also suggest that these
cells are able to induce any other cells, which did not acquire
this cytoplasm but happen to come into close contact with
these "primary" muscle cells during cleavage, to also under-
go α-actin gene activation. These would be "secondary" me-
soderm cells. According to this view, an important mecha-
nism by which α-actin genes are activated would be the ac-
quisition by cells of subequatorial cytoplasm, present in one-
cell eggs, and possibly formed during the cytoplasmic rear-
rangements that take place soon after fertilization (20). Induc-
tive cell interactions would not be involved in this
process but would have an important role in ensuring that
any other cells that are not endowed with subequatorial cy-
toplasm but that happen to be subsequently included in the
equatorial region of a blastula would also have their α-actin
genes activated. This would ensure homogeneity of tissue
differentiation. This idea is consistent with, although not
tested by, the blastomere transplantation experiments of
Gimlich and Gerhart (32).

Our results provide useful background information for fu-
ture attempts to identify molecules in eggs that lead to the
eventual activation of muscle-specific genes.

We are most grateful to Drs. J. C. Gerhart, R. L. Gimlich, P. D.
Nieuwkoop, K. Yamana, K. Shiokawa, J. Cooke, and J. Slack
for comments on the manuscript.

2. Whittaker, J. R. (1979) in Determinants of Spatial Organiza-
tion, eds. Subtelny, S. & Konigsberg, I. R. (Academic, New
York), pp. 29-51.
5. Smith, L. D. & Williams, M. (1979) in Maternal Effects in De-
6. Gurdon, J. B. (1977) in Methods in Developmental Biology,
75-84.
7. Nieuwkoop, P. D. & Faber, J. (1956) Normal Table of Xen-
pus laevis Daudin (North-Holland, Amsterdam).
9. Mohun, T. J., Brennan, S., Dathan, N., Fairman, S. & Gur-
74, 221-234.
499-504.
2, 216-224.
27. Wilson, E. B. (1925) The Cell in Development and Heredity
Fresh-Water Invertebrates (North-Holland, Amsterdam).
407-412.
24 (3), 145-158.
56, 355-360.