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Actin genes in *Xenopus* and their developmental control

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SUMMARY

The results summarized here have established the temporal and regional activation of three kinds of *Xenopus* actin genes. The cardiac and skeletal muscle actin genes are among the first cell-type-specific genes to be expressed in early development. The first transcripts to be synthesized by these genes appear to be correctly initiated, spliced, and at once translated into proteins. Both cardiac and skeletal actin genes are strongly transcribed in the axial skeletal muscle of embryos.

The mechanism by which the cardiac actin gene is first transcribed in only the somite region of an embryo depends, at least in part, on materials already localized in the subequatorial region of a fertilized but uncleaved egg. Cells which acquire this material seem able to activate their cardiac actin genes without requiring normal contact with other cells.

INTRODUCTION

The experimental work summarized here is addressed to two questions concerning the developmental regulation of genes. What activates genes at the appropriate stage in development (temporal control) and in the appropriate part of an embryo (regional control)? We have chosen to concentrate our attention on amphibian actin genes for several reasons. Actin genes are among the first, if not actually the first, identified genes (i.e. ones which make an identified product) to be activated in amphibian development and in the development of most other animals, in a region-specific way. Amphibia, while lacking the genetic advantages of *Drosophila* and mice, have enormous eggs and this makes them more satisfactory for embryological micromanipulation than eggs of other species. Several kinds of *Xenopus* genes have already been studied in considerable detail, and some of these are cell-type specific in their expression and therefore regionally controlled. These include the adult and larval globins (Patient, Kay & Williams, 1980; Patient *et al.* 1982) and also vitellogenin (Wahli, Dawid, Ryffel & Weber, 1981). The disadvantage of these genes for our purposes is that they are normally expressed only in adults or in relatively advanced tadpoles, and it could therefore be very complicated to trace the mechanisms of their activation back to the beginning of development. By choosing to work with genes strongly expressed in muscle, the first cell type to

Key words: actin, α-cardiac, actin, α-skeletal, actin, cytoskeletal, cytoplasmic determinant, gradients, induction, *Xenopus*.

undergo specialization in amphibian (and many other animals') development, we hope to reduce the number of steps which have to be understood. Our ultimate aim is to explain how the single-celled egg forms a multicellular embryo, only certain cells of which will activate a gene at the correct time.

XENOPUS ACTIN GENES AND THEIR PROBES

The first requirement in an investigation of this kind is a specific probe for each gene being investigated. Our initial work on the development of gene-specific probes and on the sequencing of cDNA has been summarized by Mohun *et al.* (1984*a*; 1984*b*). We took advantage of the fact that actin proteins are very highly conserved within the Vertebrates (Buckingham & Minty, 1983). A cDNA library was prepared from the polyA$^+$ RNA of *Xenopus laevis* gastrulae. This was screened with a cloned chicken actin cDNA. *Xenopus* cDNA clones that were positive, due to homology in the coding region, were sequenced. This revealed, as predicted from previous work with actin cDNAs of other species, that the 3' untranslated regions of the cDNAs sequenced differ substantially. Various regions of the cDNAs were transferred to M13, and these M13 probes tested on RNA extracted from several adult organs of *Xenopus*. On this basis, probes were classified as being specific for adult heart (cardiac actin) or adult leg muscle (skeletal actin). These assignments agreed with sequence results, which also permit the coding region to be classified as cardiac, skeletal, or other types. We also obtained a probe which recognizes cytoskeletal actin mRNA, again on account of its 3' untranslated region.

Most vertebrates appear to have six types of actin genes: two cytoskeletal (referred to as β and γ) and four muscle types. Preliminary Southern analyses of genomic DNA hybridized with our probes suggests that the *Xenopus laevis* genome contains only one or two genes for cardiac actin, and one or two for skeletal actin. We think that our cytoskeletal probe recognizes both β and γ actin genes.

THE TEMPORAL ACTIVATION OF ACTIN GENES IN NORMAL DEVELOPMENT

The availability of probes for cytoskeletal actin mRNA and for two different muscle actins enables us to determine the time and region of activation of these genes.

Eggs and early embryos contain mRNA for cytoskeletal actin but not for cardiac or skeletal muscle actin. This is evident from S1 nuclease protection analysis of extracted RNA (Mohun *et al.* 1984*a*, 1984*b*; Gurdon *et al.* 1984*b*), and also from a 2D gel analysis of proteins synthesized *in vitro* from extracted mRNA (Ballantine, Woodland & Sturgess, 1979; Sturgess *et al.* 1980). This last type of analysis distinguishes β and γ cytoskeletal actins, and shows that egg and early embryo RNA contains a large amount of β and a very small amount of γ actin mRNA. This implies that β and γ cytoskeletal genes are transcribed during oogenesis, the γ gene(s) being less transcribed, or the products less stable, than β gene(s). The muscle actin genes

(α) appear not to be transcribed during oogenesis or in early embryos. The confidence with which we can say this is limited in two respects. First, if the transcripts are unstable they would not have been detected. Second, the following calculation shows that even if a single-copy gene is transcribed at a maximum rate for the whole of oogenesis, and if its transcripts are stable, the accumulated transcripts would be just sufficient to be seen in a typical transcript assay. The greatest rate of transcription of a *Xenopus* gene transcribed by polymerase II is believed to be 10 transcripts/gene/min (Sargent & Dawid, 1983; Ng, Wolffe & Tata, 1984); a single copy gene, with four copies therefore in a tetraploid oocyte, would accumulate 2×10^7 transcripts in a year, a reasonable estimate of the total duration of oogenesis. With a continuously labelled M13 probe, it would normally be possible to detect between 10^6 and 10^7 transcripts by S1 nuclease protection analysis. It is therefore very likely, though not yet conclusively shown, that the cardiac and skeletal actin genes are inactive during oogenesis. The degree of uncertainty that exists, in this case, applies equally well to all other single-copy cell-type-specific genes in *Xenopus* (Perlman, Rosbash & Ford, 1977; Schafer *et al.* 1982).

A major transcriptional activation of actin genes takes place at the end of gastrulation (Mohun *et al.* 1984*a*). This applies to cytoskeletal genes (β and/or γ since our probe probably does not distinguish these) as well as to cardiac and skeletal muscle actin genes. The same stage of activation of muscle genes is seen by S1 nuclease protection, which recognizes any transcript containing 200–300 nucleotides in the 3' untranslated region, by Northern analysis, which recognizes only full-length transcripts, and by *in vitro* translation of mRNA. We therefore conclude that when these genes are first transcribed, they make correct transcripts which are fully and efficiently processed and translated. This therefore appears to be a case of a rather simple gene activation at the transcriptional level uncomplicated by regulation at various post-transcriptional levels.

The mechanism of temporal activation of actin genes is at present obscure. A simple idea would be that an inhibitor of actin gene transcription is diluted out or sequestered by the increasing amount of chromosomal DNA synthesized during early embryonic cell division. However this seems improbable in this case, because cell number increases by only twofold between early gastrula (30 000 cells at stage 10) and early neurula stages (60 000 cells at stage 15), as deduced from Dawid (1965) and Shiokawa & Yamana (1979); yet gene activation occurs rather precisely between stages 12 and 13. We are therefore left with the rather vague thought that actin gene activation depends on some previous developmental event. It is known that some genes in *Xenopus* are first transcribed at the late blastula and early gastrula stages (Sargent & Dawid, 1983; Dawid, this symposium), and the products of these might play some part in the subsequent temporal activation of actin and other genes.

THE REGIONAL ACTIVATION OF ACTIN GENES IN NORMAL DEVELOPMENT

In adults, cytoskeletal actin RNA is present in substantial amounts in all those

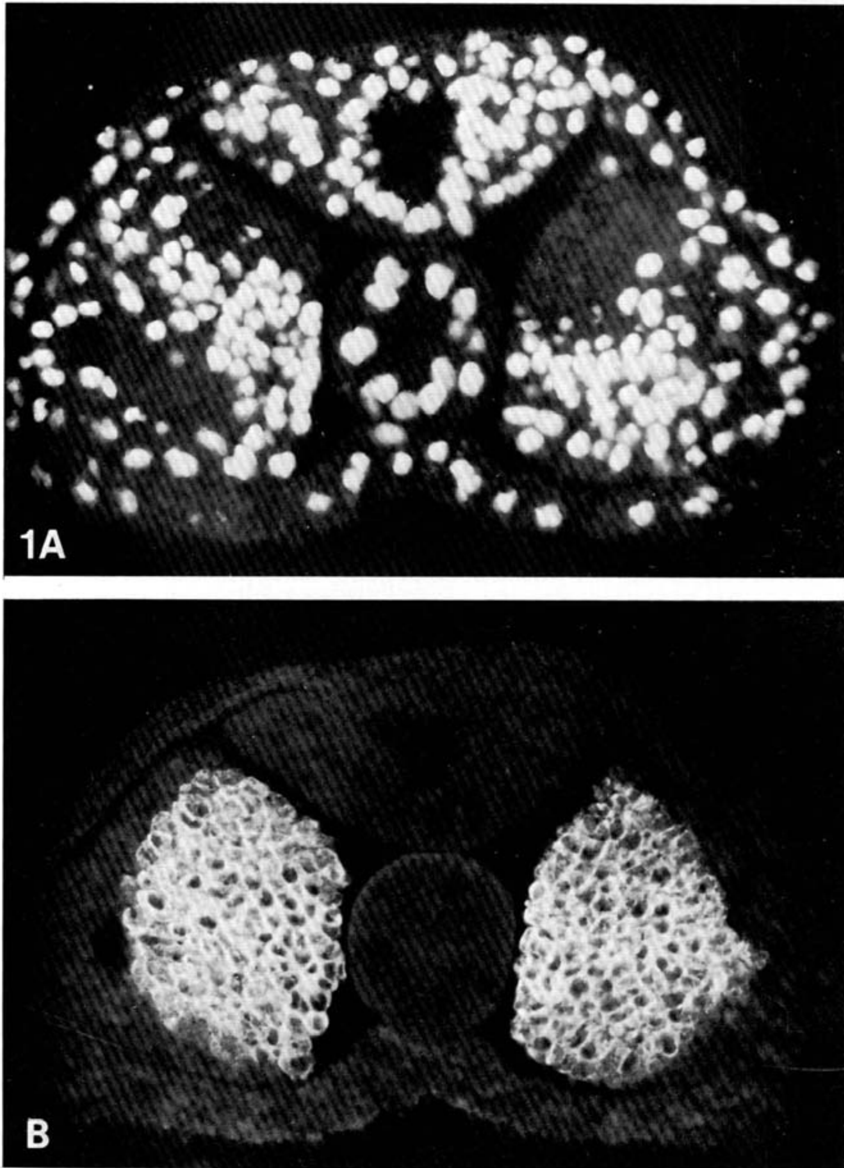

Fig. 1. Transverse section of a stage-19 neurula, treated with Hoechst which mainly stains nuclei (A), and the muscle-specific antibody 12/101 (B). Cells positive for antibody binding are restricted to the somite region. Samples were prepared as described by Gurdon *et al.* (1985*b*), and the antibodies (generously given by Drs J. Brockes and H. Gordon) applied as described by Kintner & Brockes (1984).

tissues tested (Mohun *et al.* 1984*a*); these include liver, intestine, testes, brain, kidney, and heart. In leg muscle, the amount of cytoskeletal RNA is reduced by at least ten fold. This result is in agreement with work on mammalian actins, where it is generally observed that cytoskeletal actins are reduced when muscle actins are

strongly expressed (Buckingham & Minty, 1983). In adult tissues, the cardiac and skeletal actin genes are transcribed to a detectable extent in only heart and skeletal muscle respectively, as has been mentioned, and as would be expected from other work.

In embryos, cytoskeletal actin RNA is present to roughly the same extent in the various regions tested, whereas the cardiac and skeletal genes are expressed only in embryonic muscle (Mohun *et al.* 1984*a*). This conclusion comes from the analysis of dissected regions of neurula embryos. Of six regions tested only the somites were positive for cardiac and skeletal actin RNA; even the notochord, also of mesodermal origin, was clearly negative for muscle actin RNA. The following conclusions can be drawn from this work. First, the muscle actin genes are accurately localized in their expression within a few hours of their transcripts first being detectable. Second, cardiac as well as skeletal muscle actin genes are strongly expressed in skeletal muscle of embryos. Indeed the heart is not yet present even in rudimentary form in neurula embryos. It may be that all muscle actin genes are activated in the skeletal muscle of an early embryo, to enable muscle formation to take place, and hence motility to be achieved, as soon as possible. Third, we calculate that cardiac and skeletal muscle genes must be transcribed at about the maximum rate, as soon as they are activated. Thus, if 5 % of the cells of a neurula (stage 18) have had their cardiac actin genes transcribed at 10 transcripts/gene/min since activation at stage 12, and if these are single-copy genes, then this would generate $2{\cdot}5 \times 10^7$ transcripts. This is about the amount of cardiac actin RNA which we estimate from our S1 nuclease assays to be present at stage 18 (Gurdon *et al.* 1984*b*).

More precise information on exactly which cells of an embryo transcribe cardiac and skeletal actin genes would derive from *in situ* hybridization. We are not convinced that this technology yet works well enough with yolky amphibian embryo cells to give resolution at the individual cell level. We have however been able to make use of a muscle-specific antibody, generously donated by Drs J. Brockes and H. Gordon, which is highly specific for *Xenopus* embryos muscle cells. The antibody is believed to recognize α-actinin (Kintner & Brockes, 1984). When applied to stage-18 embryos it labels only cells in the somite region (Fig. 1). From serial transverse sections of stage-18 embryos we have been able to determine approximately the number of positive cells, and hence the percentage of cells in an embryo which expresses muscle genes. This is not more than 5 %. If we assume, and this is not of course proved, that the cells positive for antibody binding are the same ones as are positive for cardiac and skeletal actin transcripts, then we have additional justification for claiming the early localization of muscle actin gene transcription, and for our calculation in the preceding paragraph.

THE MECHANISM OF REGIONAL ACTIVATION

Mechanisms that have been proposed to account for how genes are activated at the beginning of development in some cells but not others fall into the three main classes shown diagrammatically in Fig. 2. The mechanisms are shown in their

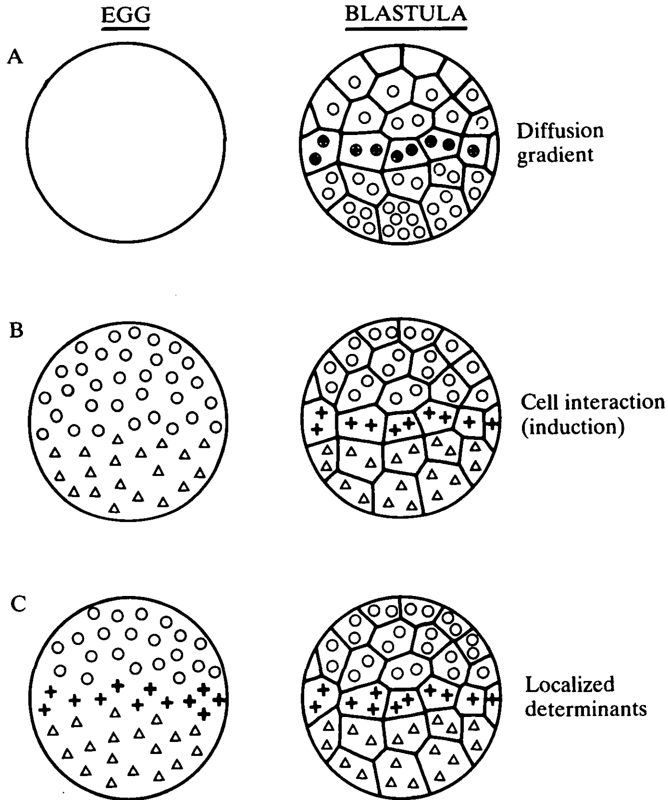

Fig. 2. Diagrams illustrating three concepts for the origin of differences between cells. In the diffusion gradient model (Fig. A), it is assumed that molecules diffuse from the vegetal (lower) to the animal (upper) poles of a blastula, forming a decreasing concentration gradient, and that cells with a middle value of this concentration, marked ⊕, are committed to mesoderm formation. In the cell interaction model (Fig. B), the egg is supposed to contain animal (○) and vegetal (△) substances; when the egg has become a blastula, cells containing each substance interact to form future mesoderm cells (+). In the localized determinant model (Fig. C), the egg is represented as already containing localized substances (+) which commit cells acquiring these to subsequently form mesodermal cell-types.

simplest form; each can be made more complicated, though in our opinion less useful, by adding further conditions under which they are supposed to operate. To which of these three mechanisms does the regional activation of muscle actin genes most closely approximate?

A prediction of the diffusion gradient model is that if part of an embryo is removed, the original gradient will be restored in the remaining part of the embryo. It is usually supposed that only lower values in a gradient can be regenerated from higher values in what is left. In a *Xenopus* embryo, we assume (as illustrated in Fig. 2A) that the vegetal pole is the highest point, and the animal pole the lowest point, in an imaginary gradient, as described by Slack (1983). If the animal region of a mid-blastula is removed, it would be predicted that the residual equatorial and vegetal

parts of the embryo would form epidermal and neural structures, even though these are normally derived by lineage from animal hemisphere cells. When the animal region of a mid-blastula is removed, and the equatorial region cultured, no obvious nervous system is formed (see Gurdon, Brennan, Fairman & Mohun, 1984*a*). Similarly the vegetal four cells of an 8-cell embryo, when separated from the animal four cells, did not form normal neural structures (Kageura & Yamana, 1983). At present we know of no good molecular markers for neural differentiation, and it may turn out, when these exist, that some neural differentiation takes place in these cases. However, the diffusion gradient model does not at all readily fit the results of the blastomere separation and blastula cutting experiments so far carried out.

The cell interaction (or induction) model of the origin of the mesoderm (including muscle) is shown diagrammatically in Fig. 2B. According to this idea, a fertilized egg consists of two phases, animal and vegetal. At the early blastula stage, it is supposed that the vegetal cells 'induce' animal cells to become mesodermal. This concept has been supported experimentally by Nakamura, Takasaki & Ishihara (1970) and by Nieuwkoop and colleagues (review in Nieuwkoop, 1977). We believe that this mechanism does indeed contribute, in normal development, to the formation of muscle and other mesodermal structures, and is discussed elsewhere (Gurdon, Fairman, Mohun & Brennan, 1985*b*).

According to the third model of cell differentiation (Fig. 2C), determinants exist in a fertilized egg and are already localized at this stage or very soon after. It is thought that whichever cells acquire these substances during cleavage are thereby committed to become mesodermal and some of them to become muscle. We have tested this model in respect of muscle actin gene activation by ligating fertilized but uncleaved eggs into two parts. Any fragment which happens to include a pronucleus divides and forms a multicellular mass which can be cultured until the normal time of muscle gene activation, and then assayed for the presence of muscle actin RNA. Fertilized eggs can be ligated in relation to the animal–vegetal or to the dorsoventral axes, since these can be recognized by the animal hemisphere pigmentation, and by the sperm entry point. As a result of these experiments, we have concluded that all components which are necessary for muscle actin activation are localized in the subequatorial region of a fertilized egg (Gurdon, Mohun, Fairman & Brennan, 1985*a*). Since assays on equivalent samples vary within a factor of two, we cannot exclude some contribution, in normal development, from the materials in the animal half of an egg, but if so, these are not essential for gene activation to take place, and are also insufficient in the absence of vegetal cytoplasm to activate genes.

This result is consistent with both the localization and the cell interaction models of cell differentiation, since it could be argued that the subequatorial region of egg cytoplasm is heterogeneous, consisting of both inducing and responding cells which interact at some time during cleavage. We have two reasons why this does not appear to be a satisfactory explanation of the egg ligation results. One comes from blastomere separation experiments. We find that the vegetal four, but not the

animal four, blastomeres of an 8-cell embryo will synthesize muscle actin RNA. This says that muscle-determining components are localized in the subequatorial region of an 8-cell embryo, as they were in the egg. We also find that the removal of the eight most vegetal blastomeres (the fourth tier) of a 32-cell embryo does not prevent muscle gene activation. From this we deduce that muscle-determining components must be localized in the vegetal half of an 8-cell embryo, but only in the upper part of this half, equivalent to the third tier of eight cells in a 32-cell embryo. It seems unreasonable to suppose that this third tier material is subsequently partitioned into a lower inducing, and upper responding, layer of cells which must interact with each other. Certainly the simplest interpretation of these results is that the third tier material confers on some of these cells a capacity to self-differentiate into muscle, without the need for a cell interaction.

A second type of experiment argues in the same direction. This is one in which we incubate eggs in Ca^{2+}/Mg^{2+}-free medium. Under these conditions, daughter cells lose adhesion from each other after each division, and a loose heap of cells is formed, unattached to each other. Miyahara, Shiokawa & Yamana (1982) have measured DNA, RNA and protein synthesis in such preparations, and have found that dissociated cells of this kind divide coordinately with normal embryos, and are similarly active in RNA synthesis. Sugimoto, Hage & Blueminck (1982) have also shown in similar preparations of cells that structural and dye transfer connections between cells, including gap junctions, are eliminated by dissociation. We have found that a fertilized egg may be kept in Ca^{2+}/Mg^{2+}-free medium up till the time when control fertilized eggs have reached stage 10, and, so long as Ca^{2+} and Mg^{2+} are added at this time, muscle gene activation takes place at the usual time (Gurdon *et al.* 1984*a*). It seems clear that much gene activation does not depend on normal contacts and communication between cells during the whole of the cleavage period. It could still be argued that an induction takes place as soon as the dissociated cells are brought into contact with each other. This seems improbable because at stage 10 most animal (or ectoderm) cells are not in contact with vegetal (endoderm) cells, and in any case would normally be induced, just after this stage in normal development, to become neural tissue following contact with the underlying mesoderm. A more satisfactory explanation of the need to supply Ca^{2+} and Mg^{2+} at stage 10 is that cells lose viability if cultured for a long time in the absence of these ions. Indeed we have observed that cells maintained in Ca^{2+}/Mg^{2+}-free conditions until stage 11 are no longer able to reaggregate after Ca^{2+}/Mg^{2+} addition.

We have tried to confirm our conclusions using mixtures of dissociated cells. If blastula equatorial cells are truly committed to undergo muscle gene activation in the absence of cell interactions, they ought to do so even if surrounded by other cells which are not committed to activate these genes. In this case, equatorial cells should transcribe cardiac actin genes to about the same extent if surrounded by one to four embryos' worth of animal or vegetal cells. The converse view is that equatorial cells are a mixture of animal and vegetal cells which must interact to create mesodermal cells able to transcribe muscle genes. In this case the addition of either animal or

Table 1. *The effect of added animal or vegetal cells on cardiac actin gene transcription by equatorial cells of blastulae.*

Cell preparation	Added cells	Cardiac actin RNA relative to controls
1 whole embryo	None	100%
1 whole embryo	None	45%
1 equatorial	1 equatorial	78%
1 equatorial	1 animal	58%
1 equatorial	4 animal	28%
1 equatorial	1 vegetal	94%
1 equatorial	4 vegetal	42%
1 animal	1 animal	0%
1 vegetal	1 vegetal	0%

All preparations were from stage-8 midblastulae. These were divided into animal, equatorial, and vegetal parts; each part was dissociated in Ca^{2+}/Mg^{2+}-free medium, thoroughly mixed with the cells to be added (also dissociated), and then allowed to reaggregate 30–60 mins later by addition of Ca^{2+}/Mg^{2+}. Reaggregates were cultured until controls had reached stage 19–20, when extracted RNA was assayed with a probe which recognizes cardiac and cytoskeletal actin RNAs as separate bands on a gel. The separation of blastulae into three regions and the RNA assays were as described by Gurdon *et al.* (1984a). The percentage values are the average of densitometer readings of bands on two gel autoradiographs. Values for cytoskeletal RNA are only approximate due to faintness of the gel band. They were all the same within a factor of 3. The numbers under cell preparations and added cells indicate the numbers of embryos used: e.g., 4 = cells from 4 embryos.

vegetal cells in excess would be expected to increase muscle gene activation, by supplying more of whichever cell-type is limiting in normal development. As can be seen from Table 1, there is a two-fold variation in the results of actin RNA assays among equivalent samples (compare the two whole embryo assays). Whether the values for cardiac actin RNA are corrected for the amount of cytoskeletal actin RNA (a measure of recovery and amount of material) or not, there is little if any effect of adding animal or vegetal cells to equatorial cells; the four-fold excess of animal cells has only a marginal effect. The interpretation of these results is complicated by the possibility that cells may rearrange themselves after reaggregation and that both mechanisms may operate in this region of an embryo. Nevertheless, if equatorial cells are solely a mixture of animal and vegetal cells which must interact to activate muscle actin genes (as in Fig. 2B), the addition of excess animal or vegetal cells should make a large difference. The results listed in Table 1 are fairly close to what would be expected if some equatorial cells are already committed to turn on muscle genes without requiring an interaction with animal or vegetal cells.

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DISCUSSION

Speaker: J. Gurdon (Cambridge):

Question from J. Slack (ICRF, London):
You suggest that the mesoderm or at least the muscle, is formed partly by a determinant in the egg, and partly by induction. But there may be a single mechanism underlying these apparently different phenomena. There are certainly models on paper in which a positional coding (the determinant) could spread autocatalytically from cell to cell and thus appear to arise also by induction.

Answer:
I agree with your interpretation which seems very reasonable, but I would say that our results are not in agreement with the *pure* induction hypothesis, namely, that the only way you can turn those actin genes on is by vegetal cells instructing animal cells to do so.

Question from J. Cooke (NIMR, London):
It seems a bit more of a problem than that to me, since there must be a very large population of cells which inherit the primary ability from the third tier at the 32-cell stage but don't go on to make muscle cells?

Answer:
This critically hinges, as you well know, on the question of just where do those muscle cells come from in normal development. I know your own results which say that they come from tier 2. One must however remember that all the published papers say they come from tier 3. Would you settle for half from tier 2 and half from tier 3?

J. Cooke:
We might hear some contributions at the workshop later. [In the Workshop, Anna Smallcombe presented results from FLDx injections into the animal tier of the 8-cell stage which gave extensive labelling of somites — Editor].