Muscle gene activation by induction and the nonrequirement for cell division

J. B. GURDON AND S. FAIRMAN

CRC Molecular Embryology Research Unit, Department of Zoology, Cambridge CB2 3EJ, UK

INTRODUCTION

In amphibia, as in many other animals with free-swimming larvae, muscle is one of the first differentiated cell types to be formed in early development. In Xenopus, the first contractions of axial body muscle take place about 30 h after fertilization, but genes required to form muscle are activated long before this, during gastrulation. Muscle actin proteins are first seen to be synthesized at the early neurula stage (Sturgess et al. 1980). More recently Mohun et al. (1984), using cloned cDNA probes, have found that cardiac actin, the type of muscle actin characteristic of adult heart, is a major component of the larval axial muscle. Xenopus cardiac actin gene transcripts are detected by S1 nuclease and Northern analysis at the early neurula stage (Mohun et al. 1984), and the use of SP6 probes on poly(A)+ RNA enables cardiac actin transcripts to be seen as early as the midgastrula stage (Cascio & Gurdon, 1986). The cardiac actin gene is transcribed in only those cells that will subsequently form muscle, and appears to be transcribed at a maximum rate as soon as the gene is activated (Gurdon, Mohun, Brennan & Cascio, 1985c). This gene is therefore especially suitable for an analysis of the mechanisms by which genes are activated in a region-specific way at the beginning of development.

In most of our work, we have used a probe complementary to the 3' untranslated region of cardiac actin mRNA. This provides a quick, sensitive and quantitative assay for transcription of this gene. We have also made use of a monoclonal antibody against another muscle-specific protein, not yet identified. This antibody, described as 12/101 by Kintner & Brockes (1984), makes it possible to determine in sectioned material which individual cells are expressing muscle-specific genes. We assume, from a comparison of antibody-treated sections with probe analysis of carefully dissected early embryos, that the same cells express both these muscle-specific genes.

Key words: muscle, activation, induction, cell division, Xenopus.
BACKGROUND

Ligation experiments with fertilized but uncleaved eggs of *Xenopus* have demonstrated that all materials required for the eventual formation of muscle are localized in a subequatorial region (Gurdon, Mohun, Fairman & Brennan, 1985a). The cytoplasm in this region of an egg is located, after five cleavage divisions, in the third tier of a 32-cell embryo; this also contains all components needed for muscle differentiation, as judged by blastomere deletion experiments. We suggested (Gurdon et al. 1985a) that some muscle cells of a larva may be formed by the activity of 'determinant' substances localized in the undivided egg, and partitioned by cleavage to the third tier of a 32-cell embryo. These hypothetical determinants would enable cells inheriting them to selfdifferentiate into muscle without the need for induction. This concept is supported by the blastomere transfer experiments of Gimlich (1986), in which transplanted dorsal tier-3 cells of a 32-cell embryo contributed to muscle when inducing a secondary axis. It is, however, possible to argue that the mechanism by which tier-3 cells contribute muscle involves an inductive process rather than the distribution of egg determinants; this would require that some daughters of a tier-3 cell should induce other daughters of the same tier-3 cell to form muscle. This seems more complicated than supposing that tier-3 cells can selfdifferentiate into muscle (Gurdon et al. 1985a).

Whatever the mechanism by which tier-3 cells of a 32-cell embryo give rise to muscle, it is clear from lineage studies that cells derived from tier 2 also contribute to muscle (Cooke & Webber, 1985) and that they do so by an induction process. It was first established by the recombination experiments of Nieuwkoop (review, 1977) that some cells from the animal one third of a blastula will differentiate into muscle (and into other axial cell types) in response to an inductive stimulus from vegetal cells of a blastula. It is hard to be sure what proportion of the larval axial muscle derives from tier-2 cells, rather than from tier-3 cells, but it is likely that at least 50% normally comes from tier 2 by induction.

We analyse this induction process with a standard design of experiment, in which a blastula is cut into three parts. The equatorial region containing derivatives of tier 3 is rejected. The animal one third (mainly tier-1 cells) is placed in contact with cells from the vegetal one third (mainly tier-4 cells). The resulting conjugate is grown until controls have reached a neurula or postneurula stage, when it is frozen and analysed by nucleic acid probes or antibodies. Typically about 25% of the animal cells (but none of the vegetal cells) contain cardiac actin transcripts or antibody-reacting material (Gurdon, Fairman, Mohun & Brennan, 1985b).

In previous work, we have established a number of characteristics of this induction system. The signal from vegetal cells does not require normal cell contacts, since it can pass between cells dissociated by the removal of Ca$^{2+}$ and Mg$^{2+}$, so long as inducing and responding cells are adjacent in a loose heap (Gurdon, Brennan, Fairman & Mohun, 1984). The need for close proximity of inducing and responding cells was confirmed by Sargent, Jamrich & Dawid
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(1986), who showed that muscle actin gene transcription was prevented, but other kinds of gene activation permitted, when dissociated cells are widely dispersed in a culture dish. We found that the ability of cells to induce and respond is strictly limited in time, vegetal and animal cells losing these capacities at the late blastula and early gastrula stages respectively (Gurdon et al. 1985b). Similar conclusions, differing in detail, were reached by Dale, Smith & Slack (1985). Contact with vegetal cells for as little as 2 h is sufficient to induce animal cells to form muscle, though the time at which animal cells start to transcribe their cardiac actin genes is always the same (late gastrula) irrespective of the time of vegetal cell contact (early, mid or late blastula) (Gurdon et al. 1985b). Thus the vegetal cells seem to give a necessary signal which can be responded to only when animal cells have passed through some event(s) related in time, like an internal clock, to the progress of normal development; thus gene activation may take place as little as 7 h or as much as 11 h after vegetal cell contact.

It is of obvious importance to identify the internal events that regulate the time when animal cells respond to vegetal cell contact. In this communication we explore the idea that one of these internal events may be cell division. The cell division that may be required for cell differentiation has been called a quantal cell cycle (Dienstman & Holtzer, 1975); this concept has received some support from many instances in different organisms where there is a correlation between cell division and cell differentiation (Reinert & Holtzer, 1975). In the particular case under discussion, it is entirely possible that cell division may be involved in animal cells' response to vegetal cells. The latest stage at which animal tissue can respond to vegetal tissue by forming muscle is stage 10\(\frac{1}{2}\) (for normal stages see Nieuwkoop & Faber, 1956). Under these conditions, the earliest time at which cardiac actin gene transcripts have been detected is 7 h later, when control embryos have reached stage 14 (Gurdon et al. 1985b). In whole embryos, cell numbers increase from about 35 000 at stage 10\(\frac{1}{2}\) to about 53 000 at stage 14 (calculated from DNA values of Dawid (1965)), and much of this increase is likely to be in the animal cells which divide more rapidly than vegetal cells. Since only a minority of animal cells activate muscle genes, it is entirely possible that each animal cell contributing to muscle may have divided after receiving a vegetal induction. The following experiments are intended to test this proposition.

INHIBITION OF CELL DIVISION

Colchicine and cytochalasin B are two widely used inhibitors of cell division. Colchicine binds to tubulin monomers, preventing them from assembling into microtubules, and inhibiting the formation of spindles. Cells undergoing division are blocked in mitosis, eventually reverting to interphase. Colchicine therefore inhibits cytoplasmic and nuclear division, and often prevents or reduces DNA synthesis. Cytochalasin B binds to actin monomers and thereby stops their assembly into microfilaments, and microfilaments already formed gradually depolymerize. Cytochalasin inhibits cytoplasmic division, but not DNA synthesis.
or nuclear division, and therefore leads to the accumulation of large multinucleate cells.

We have used these inhibitors by adding them to the culture medium in which animal and vegetal tissues were placed in contact. It did not appear to make any difference whether animal and vegetal pieces were added separately to the medium containing inhibitor and then placed in contact, or whether the whole conjugate was placed in the inhibitory medium 5 min after its formation. In all cases, cell division seemed to be arrested. The best evidence for this comes from counting cells. This is hard to do with precision, because the number of cells in an animal piece varies somewhat according to the position of cuts during dissection. As far as we can tell from cell numbers of animal pieces that have been dissociated, spread and fixed (Fig. 1A, B), cell division is arrested by each inhibitor at 10 \( \mu \text{g ml}^{-1} \) in the culture medium. Like Jones & Woodland (1986), we have incubated parts of early blastulae, whose cells are large enough to be scored visually, in these inhibitors and have directly observed cell division arrest, immediately for cytochalasin (10 \( \mu \text{g ml}^{-1} \)) and for colchicine at 100–200 \( \mu \text{g ml}^{-1} \), and in less than one hour for colchicine at 10 or 50 \( \mu \text{g ml}^{-1} \). Although colchicine does not easily penetrate the surface coat of complete blastulae, we found that it was effective when pieces of animal tissue (with no surface coat on the inside) are added, surface coat downwards, to colchicine-containing medium. It is hard to exclude a small amount of residual cell division in inhibitory media, but there is a dramatic difference between conjugates incubated with or without inhibitors (Fig. 1), and we conclude the treatment was largely effective for both responding animal and inducing vegetal cells.

The morphology of conjugates after incubation in inhibitors up till control stage 18–20 is shown in Fig. 2. Cytochalasin causes lack of normal adhesion between cells, such that they form a flattened heap (Fig. 2B). The cells in such a heap can be seen to be large, as expected if they have not divided since stage 8–9, and appear to be healthy. Some degree of adhesion exists between the animal cells of a cytochalasin conjugate, since they cannot be blown apart with a pipette, as can, for example, cells maintained in \( \text{Ca}^{2+}/\text{Mg}^{2+} \)-free medium. Conjugates incubated in colchicine at concentrations ranging from 10–100 \( \mu \text{g ml}^{-1} \) look similar to control conjugates in normal medium (Fig. 2A, C), except that their cells are larger. They appear to be healthy, as expected from the survival of \textit{Xenopus} embryos in higher concentrations of colchicine (Cooke, 1973).

**Muscle Gene Expression in Colchicine- or Cytochalasin-Inhibited Conjugates**

Conjugates cultured in inhibitors and control conjugates in normal medium were frozen at the same stage, usually stage 20, and the RNA extracted was analysed with our SP6 cardiac actin probe (Gurdon et al. 1985b). This probe identifies not only cardiac actin mRNA, but also cytoskeletal actin RNA, which has almost the same nucleotide composition as cardiac actin mRNA for about 135
Fig. 1. Arrest of cell division by colchicine. (A,B) Stage 8 animal (A) or vegetal (B) cells dissociated and photographed ¾ h later. (C,D) Stage 8 animal (C) or vegetal (D) cells dissociated, then cultured in colchicine at 10 μg ml⁻¹ until controls had reached stage 20. (E,F) Stage 8 animal (E) or vegetal (F) cells cultured as whole tissue in normal medium, without colchicine, until stage 20, when the tissues were dissociated (animal tissue with difficulty) and photographed. The figure shows that colchicine arrests cell division as judged by cell size. Colchicine at 50, 100 or 200 μg ml⁻¹ gave results indistinguishable from those shown in C and D, but appeared to be toxic (see text).
nucleotides of the probe length. The fact that the probe recognizes cytoskeletal actin mRNA in the same sample as cardiac actin RNA provides an invaluable internal control for the viability of each conjugate analysed. This is because cytoskeletal actin genes undergo a 5- to 10-fold increase in transcription during gastrula and neurula stages (Mohun et al. 1984), which is independent of induction. This increase is seen in isolated animal tissue, in the absence of vegetal cells, and is therefore a measure of the viability of the animal pieces (Cascio & Gurdon, 1986). If animal tissue is induced by vegetal cells, cardiac actin RNA undergoes a large increase which is of course entirely dependent on induction.

Representative analyses of RNA from single conjugates are seen in Fig. 3. The following conclusions can be drawn. Whether cell division is inhibited by colchicine or cytochalasin B, cardiac actin RNA is nearly always seen, though its amount is much reduced compared to noninhibited control conjugates. However, the reduction in cardiac actin RNA is matched by a reduction in cytoskeletal RNA in the same samples. At concentrations of colchicine higher than 10 μg ml⁻¹, we have seen no cardiac actin RNA, but the same samples also lacked cytoskeletal RNA and were probably dead. We conclude that the cell division inhibitors reduce all transcription, but that cardiac actin transcription resulting from induction is no more reduced than cytoskeletal actin RNA synthesis. Analyses of several samples and controls have been quantified and are listed in Table 1. It is evident from these values that whenever the cardiac actin band is low, the cytoskeletal band is similarly reduced. Since a substantial part of the residual cytoskeletal actin RNA in the inhibited samples is likely to be of maternal origin (see Mohun et al. 1984; Cascio & Gurdon, 1986), the ratios of cardiac:cytoskeletal RNA in Table 1
underestimate the relative amount of muscle gene activation by induction. The overall conclusion from these experiments is that the inhibition of cell division does not prevent the response to induction in any specific way. We interpret the reduction in cytoskeletal and cardiac actin RNA synthesis as a generally toxic effect of colchicine and cytochalasin.

To be confident of this conclusion, we would like to be sure that muscle gene activation in the inhibited conjugates takes place in a minority of animal cells, and not in vegetal cells or in all animal cells. Using the muscle-specific antibody 12/101 referred to above, we find that although cells react only weakly with antibody, it is clearly a minority of animal cells that react, that these are in a contiguous group

![Fig. 3. SP6 nuclease protection analysis of actin RNA in conjugates, with or without inhibition of cell division. An: Veg conjugates were incubated in medium containing inhibitors at 10 μg ml⁻¹, as described in the legend to Fig. 2. An: An control (lane K) indicates a conjugate of two animal pieces (in which no muscle induction takes place). The SP6 probe is protected by cardiac actin RNA for most of its length (285 nucleotides) and for part by cytoskeletal actin RNA (135 nucleotides), as described by Gurdon et al. (1985b).]
and that the group is located close to vegetal tissue, just as in control conjugates (Fig. 4).

COMPARISON WITH OTHER WORK

Jones & Woodland (1986) have described experiments similar to ours in respect of an epidermal gene expression in *Xenopus* embryos, recognized by an antibody. They found that epidermal differentiation is resistant to colchicine and cytochalasin, so long as exposure to the inhibitors commenced in embryos that had already developed to stage 8. In our experiments reported here we saw no reason to test cells before stage 8, since whatever result might be obtained would not affect our conclusion. We wish to emphasize the point that the animal cells that we have discussed are being redirected from their normal developmental fate (of epidermal or nerve differentiation) into muscle, and that this whole process is initiated only when they are placed in contact with vegetal cells. It is this whole process that appears, from our results, to be independent of cell or nuclear division.

We are not aware of other inductive systems in development that have been tested for their dependence on cell division. This has, however, been done in several cases where cell differentiation is related to egg cytoplasmic substances, especially in ascidians (Whittaker, 1973, 1977; Satoh & Ikekami, 1981). In these cases, doses of inhibitor similar to those we have used here have been sufficiently

### Table 1. Effect of cell division inhibitors on actin gene activation

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration in medium (µg ml⁻¹)</th>
<th>Type of embryo</th>
<th>Actin gene transcripts as % of cytoskeletal value in control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colchicine</td>
<td>10</td>
<td>An: Veg conjugate</td>
<td>Cardiac: 10,10,14,14,14,14,14</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>10</td>
<td>An: Veg conjugate</td>
<td>Cytoskeletal: 9,16</td>
</tr>
<tr>
<td>None (control)</td>
<td>Whole st. 10</td>
<td></td>
<td>Cardiac: 28,23 Cytoskeletal: 16,16</td>
</tr>
<tr>
<td>None (control)</td>
<td>Whole st. 18</td>
<td></td>
<td>Cardiac: 52,24 Cytoskeletal: 24,45</td>
</tr>
<tr>
<td>None (control)</td>
<td>An: Veg conjugate</td>
<td></td>
<td>Cardiac: 10,31 Cytoskeletal: 24,29</td>
</tr>
<tr>
<td>None (control)</td>
<td>An: An</td>
<td></td>
<td>Cardiac: 52,24 Cytoskeletal: 41,55</td>
</tr>
</tbody>
</table>

Animal: vegetal conjugates were prepared as described before (Gurdon et al. 1985b), and were cultured until controls had reached stage 18–20. Inhibitors were added to the medium at the time of conjugation. The values under cardiac and cytoskeletal were obtained by densitometry of appropriately developed gels of SP6 analyses. Cardiac and cytoskeletal values for the same embryo are located in the equivalent position in the Table; thus 5,5 (cardiac) and 5,7 (cytoskeletal) refers to two embryos, both of which had 5% as much cardiac actin as controls, one having 5% and the other 7% as much cytoskeletal actin. Results have been grouped according to similar values for cardiac RNA. The values under Ratio are cardiac divided by cytoskeletal.
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Fig. 4. Localization by antibody of cells in which muscle gene activation has taken place. (A) Normal medium; (B) with colchicine. A monoclonal antibody 12/101 was used as described in the text and by Gurdon et al. (1985b) on sections of conjugates incubated with (B) or without (A) colchicine at 10 μg ml⁻¹. Compared to the control (A), about the same number and location of animal cells have bound the antibody, but much more weakly.

nontoxic to permit cell differentiation or tissue-specific gene expression to appear, so long as embryos have passed through the first few divisions.

While our results seem to rule out a role for cytoplasmic or nuclear division for the inductive response, they do not exclude a possible requirement for DNA synthesis that is not inhibited by cytochalasin, and that is usually reduced but not eliminated by colchicine. We are pursuing this question.

SUMMARY

We are investigating the mechanism by which animal cells of an amphibian blastula are induced to differentiate as muscle after contact with blastula vegetal cells. After briefly summarizing previous work on this system, we have asked whether this response of animal cells to vegetal induction requires cell division. Animal and vegetal parts of a blastula were placed in contact with each other, and the resulting conjugates cultured in medium containing a sufficient concentration of colchicine or cytochalasin B to inhibit cell division. Muscle differentiation, as indicated by cardiac actin gene transcription, is induced when cell division is inhibited, though at a substantially reduced rate. However, cytoskeletal actin gene transcription, which does not depend on induction, is also much reduced under the same inhibitory conditions. We conclude that, although the cell division inhibitors seem to reduce all gene transcription, they have no preferential effect on the response to induction, and therefore that this process does not require cytoplasmic or nuclear division.
REFERENCES


