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Muscle gene activation in *Xenopus* requires intercellular communication during gastrula as well as blastula stages

J. B. GURDON, K. KAO, K. KATO and N. D. HOPWOOD

Wellcome CRC Institute, Tennis Court Road, Cambridge CB2 1QR, UK

Summary

In *Xenopus* an early morphological marker of mesodermal induction is the elongation of the mesoderm at the early gastrula stage (Symes and Smith, 1987). We show here that the elongation of equatorial (marginal) tissue is dependent on protein synthesis in a mid blastula, but has become independent of it by the late blastula stage. In animal caps induced to become mesoderm, the time when protein synthesis is required for subsequent elongation immediately follows the time of induction, and is not related to developmental stage. For elongation, intercellular communication during the blastula stage is of primary importance.

Current experiments involving cell transplantation indicate a need for further cell:cell interactions during gastrulation, and therefore after the vegetal-animal induction during blastula stages. These secondary cell interactions are believed to take place among cells that have already received a vegetal induction, and may facilitate some of the later intracellular events known to accompany muscle gene activation.

Key words: mesoderm, induction, *Xenopus*, intercellular communication, gastrula, blastula, protein synthesis, muscle genes.

Introduction

Mesoderm-forming induction in *Xenopus* is the subject of intense investigation at present, particularly in respect of growth factor-like molecules, which can cause cultured animal pole cells to undergo various types and degrees of mesodermal differentiation (Green and Smith, 1991; Jessel and Melton, 1992). Although many morphological responses of mesoderm are not observed till neurula or later stages in development, for example the formation of notochord and muscle, and the appearance of blood cells, it is generally believed that the initial receipt of inductive signals specifying the entire mesodermal lineage takes place during cleavage and is complete by the late blastula stage. Three reasons for this are the following. First, ectoderm (animal cap) tissue of *Xenopus* has lost its competence to form mesoderm by the early gastrula stage, when induced experimentally (Nakamura et al., 1970; Gurdon et al., 1985a). Second, animal cells are capable of responding to inductive signals at about stage 6½ (48 cell stage), as shown by placing early blastula animal cells in contact with vegetal tissue at the end of its inductive life (Jones and Woodland, 1987). It is therefore believed that a signal from vegetal cells starts to be received by animal cells at about the 48 cell stage and continues for about 6 hours, by which time embryos have reached the early gastrula stage. Third, the earliest responses to mesoderm induction include the transcriptional activation of the gene *Mix1*, which reaches its maximal expression by stage 10 and declines thereafter (Rosa, 1989). The transcription of several other mesoderm response genes is initiated before gastrulation. Among these are *XMyoD* (Hopwood et al., 1989; Harvey, 1991; Scales et al., 1990; Frank and Harland, 1991; Rupp and Weintraub, 1991), *Xbra* (Smith et al., 1991), and *Xgoosecoid* (Cho et al., 1991).

In this article, we first describe results concerning the timing of the inductive signal which elicits an early mesoderm-specific response, namely tissue elongation. This is an in vitro assay for the convergent extension movements of gastrulation (review by Keller, 1991). We then comment on the results of current cell transplantation experiments, which lead to the conclusion that some kinds of cell communication take place during gastrulation, and therefore after receipt of the initial inductive signal from vegetal cells.We think that these cell:cell interactions during gastrulation may be required for some subsequent mesodermal responses such as muscle gene activation.

Gastrula elongation and the inhibition of protein synthesis

Previous work (Cascio and Gurdon, 1987) showed that muscle gene activation following mesodermal induction is dependent on protein synthesis during late blastula and early gastrula stages; the reversible suppression of protein synthesis by cycloheximide at these stages prevented the initiation of cardiac and cytoskeletal actin gene transcrip-

tion. We have now extended this analysis by determining the time when protein synthesis is required for cells to undergo the elongation movement known to be a characteristic of gastrulation and a very early response to mesoderm induction (Symes and Smith, 1987; Smith et al., 1990). Although changes in single cell motility on fibronectin is an earlier response to induction (Smith et al., 1990), we have chosen to use in vitro tissue elongation as a more convenient indication of induction.

Either cycloheximide or puromycin can suppress protein synthesis when added to the medium in which parts of embryos are cultured (Fig. 1). Furthermore the inhibition is reversible, sooner after puromycin than cycloheximide. The in vitro elongation of embryo explants, which represents the normal gastrulation movements of mesoderm cells, is prevented by these same inhibitors in a dose-dependent way and is correlated with the extent of protein synthesis inhibition (Fig. 2).

To determine the stage during blastula formation at which subsequent elongation is sensitive to protein synthesis, we incubated vegetally depleted embryos in inhibitors, starting at various mid or late blastula stages. The result is that these explants are completely prevented from elongating when protein synthesis is inhibited starting at stage 8, but elongate normally if inhibition is commenced at stage 9 or later (Fig. 3).

We next asked whether the time when protein synthesis is required for subsequent elongation is related to a particular stage in development (stage 8) or to the time elapsed from receipt of an inductive signal. This was tested by exposing animal caps at stage 7 to XTC-cell inducing medium, and then treating them with puromycin at various times after that. Elongation was scored as described in Table 1 when control embryos had reached stage 13; those vegetally depleted embryos that showed elongation did so during the time when whole control embryos were gastrulating. The result (Table 1) is that animal caps did not elongate when treated with puromycin 1-2 hours after induction by XTC medium, but did so normally when puromycin was added at stage 9 or later, that is 4 or more hours after induction. However, when animal caps were exposed to XTC medium at stage 9 and then at once to puromycin, elongation was inhibited (Table 1). In conclusion, subsequent elongation requires protein synthesis immediately after induction, irrespective of the stage when induction takes place. This aspect of timing may be contrasted with that previously described for muscle gene activation (Gurdon et al., 1985a) and for elongation itself (Symes and Smith, 1987), which always take place at a particular developmental stage, independently of the time of induction, namely at the mid and early gastrula stages respectively.

We have discussed elongation as an example of an early mesoderm-specific response to vegetal induction. The animal cap experiments outlined above show that the vegetal signal leading to mesoderm induction is immediately followed by a period of protein synthesis which is required for subsequent elongation. For comparison, protein synthesis is required for the initiation of transcription of *XMyoD* (Harvey, 1991), but not for that of *Mix1* (Rosa, 1989), *Xbrachyury* (Smith et al., 1991) or *goosecoid* (Cho et al., 1991). It is important to appreciate that most of the work on early amphibian induction involves experimental combinations of animal and vegetal tissue or animal caps treated with growth factor-like substances, and that these do not necessarily reflect the timing of induction in normal development. On the other hand, the direct analysis of equatorial cells indicates the normal timing of events in those cells that contribute the great majority of the mesoderm. We find that the time when equatorial cells of a blastula normally synthesize protein required for future elongation is during stage 8. This could mean that these cells received a vegetal induction shortly before stage 8, or that they inherited cytoplasmic substances from the egg with the same effect (Gurdon et al., 1985b). In either case, it seems that equatorial cells have completed their receipt of, and immediate response to, vegetal signal before gastrulation. This agrees with the time of transcription of early response genes, as mentioned above. It will be interesting to see whether the time of protein synthesis sensitivity for *Mix1*, *goosecoid*, etc. is the same for normal equatorial cells as for in vitro induced animal pole cells.

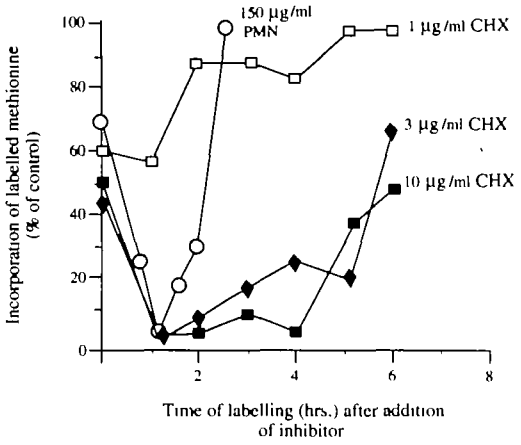

Fig. 1. Cycloheximide (CHX) and puromycin (PMN) reduce protein synthesis reversibly. Stage 8 embryos whose vegetal region had been removed to increase exposure to the medium were incubated at 23°C in various concentrations of CHX (1,3 and 10 µg/ml) or PMN (150-200 µg/ml) for one hour, and this was followed by extensive washing in MBS. By removing the vegetal one third of embryos, the animal and equatorial two thirds remained "open" and readily accessible to inhibitor and washes. Radioactively labelled methionine was added to the incubation medium of embryos for one hour at hourly intervals at the beginning of and following CHX addition (horizontal axis). The embryos were then frozen to determine the level of incorporation of label into acid-insoluble material (vertical axis). Each point represents the data collected for at least 4 embryos taken from two different batches of embryos. Values were normalized to one embryo before they were compared to the controls.

Muscle gene activation requires cell interactions during gastrulation

When a vegetal induction has been received by cells during blastula stages, it might be thought that further events, lead-

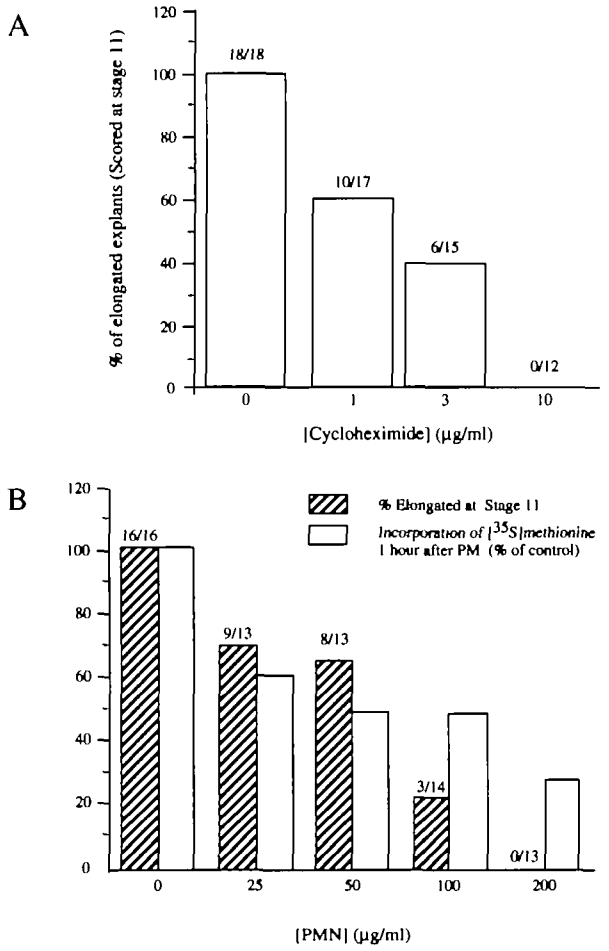

Fig. 2. Cycloheximide and puromycin prevent elongation. Embryos had their vegetal region removed at stage 8, were allowed to heal in MBS for half an hour, and were then exposed to various concentrations of CHX (A) or PMN (B). Also shown in B is the level of incorporation of [^{35}S]methionine after PMN treatment. At stage 11, the embryos were scored for elongation which was considered to have taken place when an embryo's longest dimension was at least twice its shortest diameter. Any elongation observed always took place at the normal time during gastrulation.

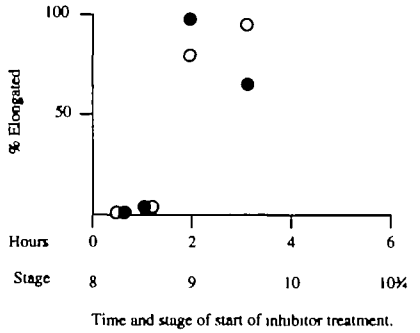

Fig. 3. The inhibition of elongation by protein synthesis inhibitors is stage-dependent. Embryos had their vegetal region removed at stage 8 and were treated with 5 μg/ml of CHX (open circles) or 200 μg/ml PMN (solid circles) for one hour starting at the times and stages indicated. Each point represents the start of a period of inhibition of protein synthesis lasting for four hours (CHX) or for two hours (PMN).

ing to muscle gene activation, are solely intracellular. The first indication that this is not the case and that further cell interactions are required came from experiments in which isolated cells were cultured with or without calcium (Gurdon et al., 1984). Late blastula cells, which had received their vegetal inductive signal, were dissociated, cultured as loose cells in Ca-free medium, and then reaggregated by Ca^{2+} addition at various times. It turned out that Ca^{2+} addition and cell reaggregation are absolutely necessary, during the early gastrula stage, for muscle genes to be subsequently transcribed, but not for the elongation or gastrulation type of cell movement (Smith et al., 1990), nor for the activation of cytokeratin genes, which takes place independently of any cell interactions (Sargent et al., 1986). Another indication that mesoderm-forming cell interactions continue during gastrulation comes from the grafting experiments of Smith et al. (1985). These authors found that ventral equatorial tissue, which normally forms blood cells and mesenchyme, will form muscle if cultured in association with dorsal equatorial tissue. They suggest that this represents a normal "dorsalization" signal taking place at stage 10, and perhaps later. All of these results imply that some interaction must take place among cells during gastrulation, and that this is different from the inductive interaction which precedes it.

Further indications that cell interactions during gastrulation are required for the full activation of muscle genes come from cell transplantation experiments. In some of these, single future muscle cells have been transplanted to a non-muscle region of other embryos. Kato et al. (unpublished) find that only when a cell is taken from a late gastrula, can it continue its differentiation as a muscle cell; if taken from earlier stages, similar cells fail to differentiate as muscle. The conclusion is that, during gastrulation, future muscle cells normally undergo some process that commits them to stable muscle gene activation. These results extend the single cell transplantation experiments of Heasman et al. (1984) by using muscle-specific gene markers with which to recognize differentiation.

Another design of cell transplant experiment using future muscle cells indicates the importance of cell interactions during gastrulation. Cells at the mid-gastrula stage are placed in sandwiches composed of blastula ectoderm, either as single cells or as reaggregates, and then cultured overnight before being tested for muscle gene expression. This is observed only in the reaggregated cells and not in single cells, a result that we interpret as indicating the need for an interaction among cells that have already embarked on a mesodermal pathway of differentiation due to events prior to gastrulation. Since the only difference between the single and reaggregated cells is the nature of their neighbours, we consider that these results point to the importance of cell interactions, during gastrulation, for muscle gene expression. These results (in preparation) extend the concept of a community effect, originally described for blastula cells in vegetal tissue sandwiches (Gurdon, 1988, 1989), to events in normal muscle cell differentiation. These

Table 1. *Elongation of animal caps requires protein synthesis immediately after XTC treatment, independently of developmental stage*

Stage of XTC treatment	Initiation of PMN treatment (Hrs after XTC)	Stage of PMN treatment	Elongation score No (%)			Total
			+	++	+++	
7	-	-	0	0	12(100)	12
7	1	8	0	0	0	10
7	2	8½	6(60)	0	0	10
7	3	9	5(42)	3(25)	0	12
7	4	9½	0	7(58)	4(33)	12
9	1	9½	0	0	0	10

XTC treatment was for 1 hour.
Puromycin (PMN) treatment was at 150 μg/ml for 30 minutes.
Elongation was scored by the ratio, at stage 11, of an embryo's longest dimension to its shortest diameter: +, 1.5-2.0, ++, 2.0-3.0; +++, greater than 3.0.

experiments and results differ in two respects from those of Godsave and Slack (1989, 1991) who described muscle cell differentiation in the progeny of single cells. First, the in vitro cultures used by these authors were initiated as single mid-blastula (stage 8) cells, each of which divided to form daughters, and hence a small group of cells; our experiments were initiated with stage 11 cells which divided little if at all. Secondly, Godsave and Slack cultured cells on a fibronectin- and laminin-coated substratum, and added gamma-globulin to the medium, procedures that might influence cell differentiation. In our experiments, implanted cells were surrounded by a normal ectodermal environment with which they are normally in contact.

Molecular events associated with the initiation of muscle actin gene transcription

For several years, our group has been analyzing the promoter of the *Xenopus* cardiac actin gene, which is strongly expressed in skeletal muscle, and, together with skeletal actin, is the first structural muscle protein gene to be transcribed in development. This gene is first expressed at the mid-gastrula stage (Mohun et al., 1984; Cascio and Gurdon, 1986), and we may ask whether any of the events associated with this might result from cell interactions during gastrulation.

Three regions of the cardiac actin promoter have been shown by deletion analysis to be of major importance in initiating its transcription during gastrulation (see review by Gurdon et al., 1992). One is an upstream GC-rich region about which very little is currently known. Another is a CArG box sequence [CC(6A or T)GG], of which there are four copies, but only the most 3′ of these centered at −85 is of key importance. Several proteins have been identified that bind to this sequence; most of these are present from fertilization onwards, and, subject to the possibility of secondary modifications, are available to bind to the actin promoter before gastrulation. These factors are reviewed by Mohun et al. (this volume).

The third significant part of the cardiac actin promoter is the M region (Taylor et al., 1991), which contains three copies of the E-box motif (CANNTG), to which proteins of the MyoD family can bind. In *Xenopus*, *XMyoD* and *XMyf5* genes are activated early in development, their mRNAs accumulating rapidly during early gastrulation, about two hours before the first appearance of transcripts of the cardiac actin gene (Hopwood et al., 1989, 1991; for review, which refers also to the work of other laboratories, see Gurdon et al., 1992). By the end of gastrulation, these mRNAs are restricted to the developing myotomes, in which the cardiac actin gene is also specifically expressed (Hopwood et al., 1989, 1991). XMyoD protein, which is known to accumulate only in myotomal nuclei (Hopwood et al., 1992), and XMyf5 protein, can bind specifically to the M region (Taylor et al., 1991). XMyoD and XMyf5 are therefore likely to be key factors in determining the specificity of cardiac actin gene transcription.

Further support for this view is provided by ectopic expression experiments, in which synthetic XMyoD or XMyf5 mRNA was microinjected into early embryos. Either kind of mRNA was able to activate transcription of the cardiac actin gene in animal cap ectoderm, which would not normally express it (Hopwood and Gurdon, 1990; Hopwood et al., 1991). Furthermore, we have recently found a threshold effect for the stable activation of muscle-specific gene expression in animal cap cells (Hopwood et al., unpublished). Below a threshold dose of XMyoD mRNA, transcription of the cardiac actin gene is only transient, but above it expression is sustained and later muscle markers are also activated. These results indicate that MyoD family members are sufficient to activate muscle-specific gene expression in normal embryonic cells, and that this activation might normally be stabilized by a threshold mechanism. However, parallel staining of XMyoD mRNA-injected animal caps and normal, uninjected future muscle cells using an anti-XMyoD monoclonal antibody showed that some injected animal cap cells contain, until the end of gastrulation, more XMyoD protein than the early muscle cells, without subsequently expressing muscle markers stably. For this reason, it is likely that other muscle-specific factors, not themselves activated by XMyoD, are required for normal muscle-specific gene activation. XMyf5 could have a significant additive, though not strongly synergistic effect (Hopwood et al., 1991), but there may be a role for other factors yet to be discovered.

For the purposes of the present article, we may point out that it is during neurulation (stages 13-18), that above-threshold animal caps have a normal myotomal concentration of XMyoD protein, and just subthreshold animal caps do not; before this stage, subthreshold animal caps have a higher concentration of XMyoD protein than normal myotome cells. One interpretation of this observation is that neurulation is the stage when cells sense, and respond to, the threshold concentration of XMyoD protein. It is also possible that the threshold concentration of XMyoD protein is sensed by animal cap cells at an earlier stage in development, when, it must also be supposed, they lack some other factor(s) needed by animal (but not future myotomal) cells to cooperate with XMyoD.

In summary, a considerable amount of information exists about genes and factors concerned with the initiation of muscle actin gene transcription. Although the association of factors with genes must involve intracellular processes, it is quite possible that the regulation of these events, such as the association of regulatory proteins with each other or with the muscle actin gene promoter, may be influenced by cell:cell interactions which take place during gastrulation.

Conclusion

The mesoderm of *Xenopus* embryos is believed to be formed as a result of a cell:cell interaction between inducing vegetal and responding animal cells. This interaction is completed by the end of the blastula stage. Since many of the responding genes, such as those that encode structural muscle proteins, do not start transcription at a significant rate until mid gastrulation or later, several events, yet to be identified, must take place between receipt of the vegetal inductive signal and the transcription of response genes. We have summarized here some of the reasons, which are still preliminary, for believing that further cell:cell interactions take place during gastrulation, and have an important role in connecting mesoderm induction with stable gene activation. These interactions might help to regulate some of the intracellular events involved in muscle gene activation, such as the association of myogenic proteins with a muscle gene promoter.

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