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Colloquium Paper

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An experimental system for analyzing response to a morphogen gradient

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ABSTRACT **A recently described experimental system for analyzing the mode of action of a morphogen gradient involves the *in situ* hybridization of sectioned tissue constructs. In these constructs, a source of activin signaling induces the transcription of several mesodermal genes in blastula animal caps, according to the position of cells in a concentration gradient. New experiments show that activin-loaded beads emit a signal for only 2 hr and that the same cell can be induced to express different genes. We determine the position in the gradient and the time after the start of activin signaling at which early genes, including *Mix1, Xpo, Xwnt8, Xchd,* and *Xlim1*, are activated, relative to the previously tested genes *Xbra* and *Xgsc*.**

The term morphogen gradient has enjoyed persistent attention in the embryological literature for many decades. It describes a situation in which a type of molecule is distributed in a concentration gradient over several cell diameters. A morphogen is a substance whose concentration is read by cells to discover their position relative to a landmark or to the source of morphogen (1). Cells usually respond to a morphogen concentration by activating or repressing a gene. An apparent example of a morphogen is retinoic acid; when supplied in a bead to the anterior region of a chicken limb bud, retinoic acid (or a metabolite of it) spreads out from that point in a concentration gradient and elicits the formation of digits, the type of which is related to the position of cells in the gradient (2). Morphogen gradients are likely to be of considerable importance in development; this is because a single event causing release of morphogen molecules from a point can initiate the formation of several cell types and structures, as cells respond in different ways to the morphogen concentration around them. There are several stages in animal development when morphogen gradients are likely to be involved. These include mesoderm (3), somite (4), and neural induction (5) in vertebrate development, vertebrate limb differentiation (6, 7), and dorso-ventral axis differentiation in *Drosophila* (8).

The eventual aim of our work described here is to understand how cells make different responses to various concentrations of the same molecule. An important advance in this direction was made by Green *et al.* (9, 10) who showed that a range of different genes can be activated by exposing dissociated *Xenopus* blastula cells to different concentrations of activin. Activin is a member of the transforming growth factor β class of growth factors, is present in *Xenopus* embryos (11), and is extremely effective, at low concentrations, in inducing blastula ectoderm (animal cap) cells of *Xenopus* to form mesodermal cell types (9). It has not yet been shown that activin is a natural mesoderm-inducing molecule. Nevertheless, activin and animal caps seem at present to provide the best experimental system for analyzing the mesoderm-forming (Nieuwkoop) induction in *Xenopus*. With this in mind, we have recently described ways of making the activin animal cap assay suitable for analysis at the cellular level (12, 13).

The purpose of this article is to summarize our results so far obtained with this system and to provide new information on previously untested genes. In this way, we hope to use this system to understand how morphogen gradients are interpreted by cells in a concentration-dependent way.

The Experimental System

The principle of our experimental design is to supply a localized source of activin that maintains a constant spatial relationship to a field of responsive cells. After a fairly short time of 2–4 hr, the preparation is fixed, and gene expression in the responding cells is assessed by *in situ* hybridization. The activin source emits a signal, presumed to be activin, in a concentration gradient radiating away from the source. Two kinds of activin sources have been used. One is an animal cap containing synthetic activin mRNA, the RNA having been injected at the two-cell stage. The other involves the implantation of beads containing activin protein between two animal caps. In the former case, the inducing and responding animal caps are distinguished by marking one of them with a fluorescent lineage marker. Fig. 1 illustrates these constructions. An important feature of our analysis is that *in situ* hybridization is carried out in serial sections of the constructs; by this means, deep and superficially located cells are equally accessible to the hybridization probe, and the distance of cells expressing various genes from the activin source can be measured directly. When comparing the pattern of expression of two genes, nearby sections from the same conjugate are mounted onto two slides and hybridized separately to a probe for each gene. To visualize hybridization, we use digoxigenin-coupled RNA probes, to which an alkaline phosphatase-carrying antibody is subsequently bound, according to Boehringer instructions.

Results Obtained So Far

A remarkable characteristic of this experimental system is that a band of gene expression can be seen to move away from the activin source as the strength of the source is increased (12), much as a ripple moves away from a pebble dropped in a pond (Fig. 2). This effect is observed with the gene *Xbrachyury* (*Xbra*; ref. 14), which responds to relatively low activin con-

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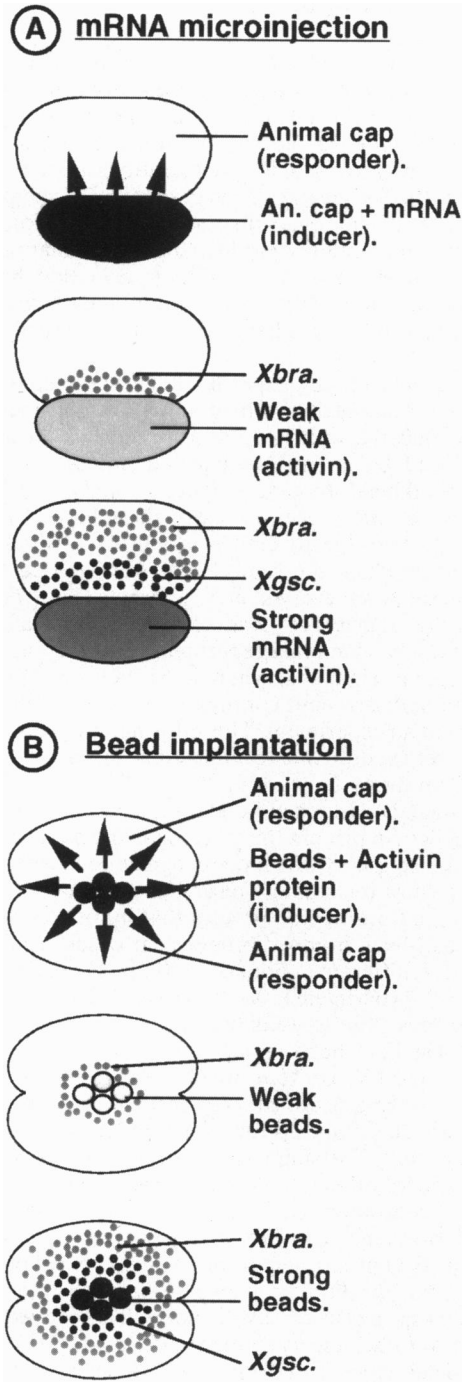

FIG. 1. Diagrams of two experimental procedures for analyzing gene activation induced by an activin source of varying strength. Arrows indicate the direction of activin signaling, and hence the direction of a progressively weakening morphogen concentration. Each animal cap contains a few thousand cells, and is taken from mid- to late-stage *Xenopus* blastulae. The constructs are cultured for 2–4 hr and then fixed, sectioned, and hybridized to probes for *Xbra* or *Xgsc*.

centrations (10, 12). On the other hand, *Xenopus goosecoid* (*Xgsc*; ref. 15) responds to higher activin concentrations (10), and its expression is observed, in our system, between the activin source and the region of *Xbra* expression (Fig. 3*A*). The only simple interpretation of this result is that the concentration gradient resulting from activin increases progressively with the strength of the source and that the particular concentration range to which *Xbra* responds moves centrifugally away as the strength of the source increases.

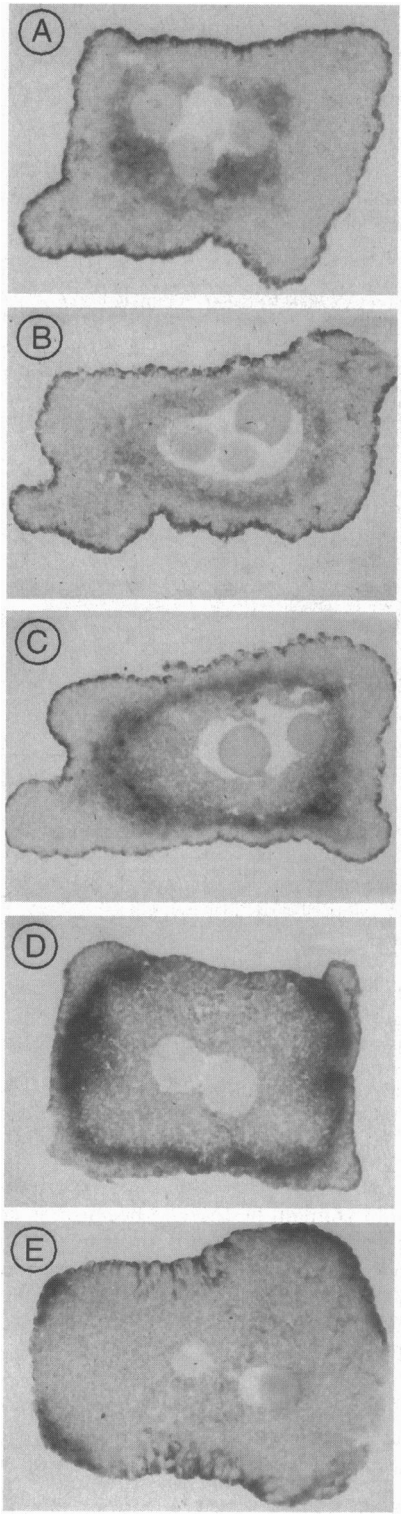

FIG. 2. A ripple of *Xbra* expression (dark band) is seen at an increasing distance from the centrally located activin-containing beads. Constructs were prepared and processed as indicated in Fig. 1*B*. Affigel blue beads (Bio-Rad) were washed, incubated in recombinant human activin in 0.1% BSA for 30 min at 37°C and then stored at 4°C for up to 1 week. Beads used were loaded in 2 (*A*), 4 (*B*), 8 (*C*), 16 (*D*), and 32 nM (*E*) activin solutions. BSA competes with activin for binding sites on beads; a reduction BSA concentration would allow much lower activin concentrations to have the same effect but gives less reproducible results. Each animal cap sandwich was loaded with five beads.

We might predict that an expanding ripple of *Xbra* expression should also be seen with increasing time after the im-

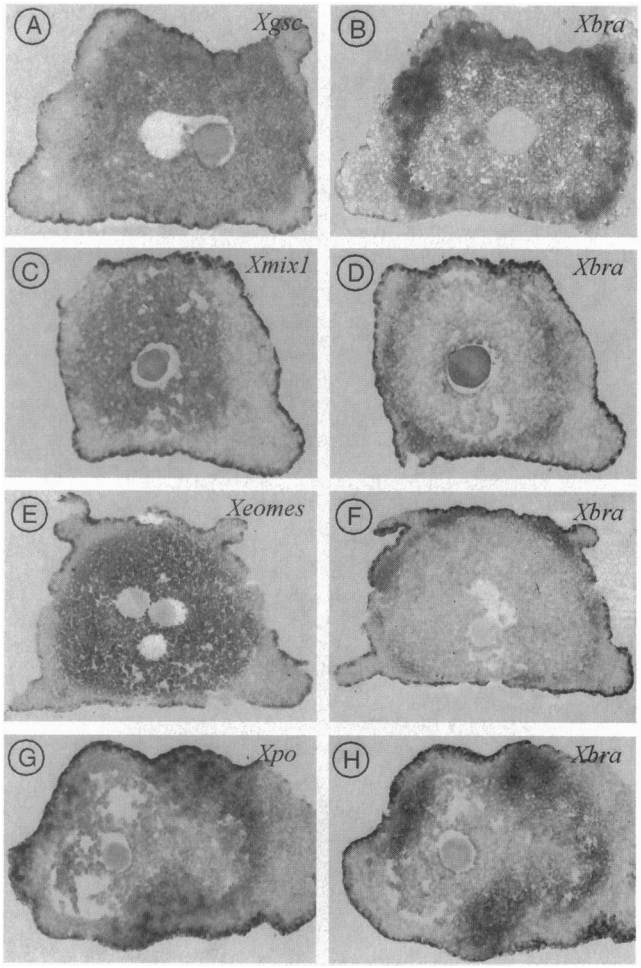

FIG. 3. High- and low-response genes. The location of cells expressing the genes indicated is shown in relation to an activin source. Each animal cap construct, prepared as described in Fig. 1*B*, contained activin beads (see legend to Fig. 2). In each case, sections were hybridized with the probe shown on the left (*A*, *C*, *E*, and *G*) and, in parallel, nearby sections from the same conjugate with an *Xbra* probe (*B*, *D*, *F*, and *H*).

plantation of strong activin beads. This experiment cannot be done as simply as might be thought because, in common with a number of developmental events, the time of *Xbra* expression is related to the developmental age of the responding animal cap cells and not to the time elapsed from bead implantation (13, 16, 17). Nevertheless, a time course of *Xbra* expression can be achieved by removing beads at intervals after implantation and culturing the disbeaded conjugates until the normal time of *Xbra* expression, at stage 10.25. When this is done, an expanding shell of *Xbra* gene expression is indeed seen (13), a result in agreement with our interpretation of an expanding gradient of morphogen.

The possibility of removing and replacing implanted activin beads at any desired time has enabled us to determine the time when cells sense the activin concentration surrounding them. We have found that cells can change their gene expression from the low concentration response *Xbra* to the higher concentration response *Xgsc* if weak beads are replaced by strong beads (13). Thus we conclude that cells can monitor and respond to activin continuously, at least over a period of a few hours. However, when strong beads were replaced by weak beads, cells did not change their response downward, a result suggesting a ratchet mechanism by which cells can readily switch to a higher level of gene response as the strength of the gradient increases.

Interpretation of Past Results

We have not been able directly to visualize a gradient of activin, or of any other molecule, in these experiments. Indeed this has not been achieved in any other experimental or natural situation for secreted molecules in multicellular tissue, probably because effective activin concentrations are in the 10–100 pM range (10). The clearly visible gradient of *bicoid* protein in *Drosophila* (18) is in a syncytium and is not therefore comparable to an activin-induced gradient in multicellular tissue. The biochemical measurements of retinoic acid that have been made after bead implantation are within a few fold of 20 nM (19), but even then a gradient has not been directly demonstrated.

Other aspects of our experimental system have become more clear as a result of the following experiments. It is noticeable that the width of the *Xbra* band is much greater when induced by an mRNA-injected animal cap than by protein-bound beads (compare figure 4 *c* and *h* in ref. 12). To investigate this difference, we tested the length of time over which beads continue to emit a signal, by a serial transfer experiment in which the same beads were moved to a new animal cap sandwich every hour. The results (Fig. 4) show that the signaling activity of beads is largely lost after 2 hr. Therefore our bead implant experiments in effect supply a 2-hr pulse of activin signaling, whereas we believe that mRNA-injected animal caps emit continuous activin signaling during the course of an experiment. This information helps us to plan and interpret experiments terminated at different times after bead implantation.

To adequately understand our results, we should know the half-life of activin protein (or other signaling molecule) and of the *Xbra* transcripts by which we recognize cell response. Since we do not know the stability of activin protein or the perdurability of the transduction process that it initiates, we cannot estimate half-lives from experiments in which beads are removed after different amounts of time. However, in bead replacement experiments, we have seen that *Xbra* mRNA located in cells close to weak beads has largely disappeared 2 hr after these have been replaced by strong beads (13). This indicates a half-life for *Xbra* mRNA of 1–2 hr. On the other hand, when strong beads are replaced by weak beads, *Xbra* RNA persists in distal cells for >2 hr, and *Xbra* is not induced in proximal cells. The simplest explanation for these results is that *Xgsc* and/or other strong response genes induced by a high activin concentration cause turnover of preexisting *Xbra* mRNA in the same cells. We have observed that when beads of a given strength are left in place for several hours or are removed after 2 hr (both providing a 2-hr pulse of signal), *Xbra* mRNA persists in cells for several hours. This can be explained in two ways. Either the signaling transduction process continues after bead removal, or *Xbra* mRNA persists for >2 hr when it is not superceded by a stronger (e.g., *Xgsc*) response.

Can the Same Cell Be Induced to Express Different Genes?

There are two routes by which a population of cells can be made to express different genes. One is where each cell has a broad choice of gene expressions; the other is where the tissue is heterogeneous, each cell being predetermined to either express or not express a particular gene. In the latter case, the various genes induced by increasing activin concentration would result from the activity of different cells within the population and not of different genes by the same cells. We believe our system is of the first kind, for the following reasons. As activin bead strength is increased from very weak to very strong, a band of *Xbra*-positive signal moves centrifugally from cells near the beads to cells in the distant periphery of the field. If preparations are *in situ*-hybridized at an early stage (stage 10.25), *Xbra* mRNA is

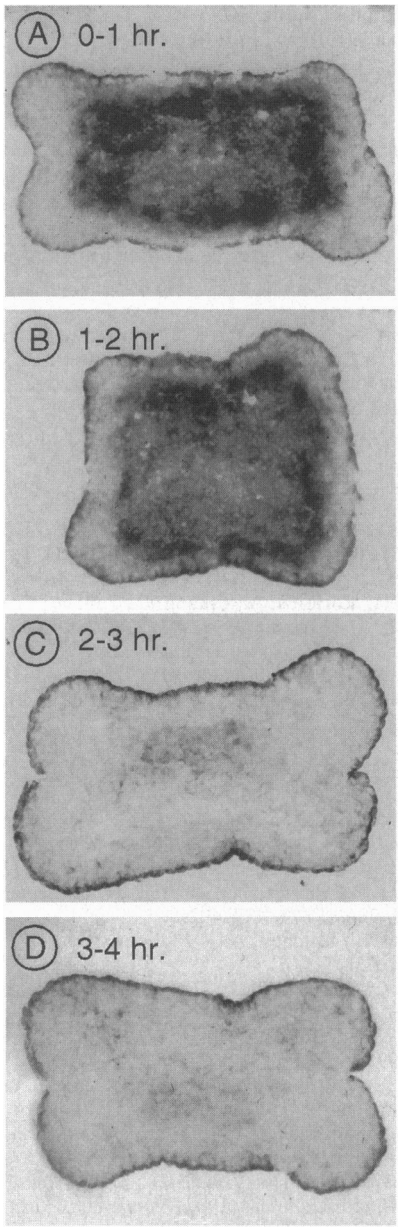

FIG. 4. Activin beads lose potency 2 hr after implantation. Activin beads (32 nM) were implanted into an animal cap sandwich, as in Fig. 1*B*. After 1 hr, the beads were removed and implanted into another sandwich, and so on (Fig. *B–D*). Disbeaded constructs were cultured until stage 10.5 and analyzed for *Xbra* expression. The beads lose potency after the second transfer.

mainly nuclear, and it can be seen that all nuclei in the band are stained by the probe. As the *Xbra* "ripple" moves outwards, with increasing bead strength, the cells between the *Xbra* band and the beads are all stained by the *Xgsc* or *Xeomes* (see below) probes. We do not see a speckled cell population, in which only some of the cells in the *Xbra* or *Xgsc* regions are stained. Eventually it will be helpful to have double staining, at the single cell level, on the same preparation, to ensure that every cell in the appropriate part of the gradient is marked by one or other probe. However, the results already obtained are inconsistent with the existence of two different types of animal cap cells capable of expressing either *Xbra* or *Xgsc*. We therefore conclude that most if not all animal cap cells can be induced to express *Xbra* or *Xgsc* genes. Since they express neither gene if not induced, there are at least three choices open to these cells.

What Is the Position of Other Gene Responses Within the Activin Gradient?

We have established that *Xgsc* and *Xbra* are activated in response to different concentrations of the presumed activin gradient. The question now arises whether other early *Xenopus* mesodermal genes respond to levels of the gradient that are different from those which activate *Xgsc* and *Xbra*.

To investigate this, we have tried to extend our method of *in situ* hybridization on sectioned conjugates so that we can map the position of expression of genes other than *Xbra* and *Xgsc*. Fig. 3 *C–H* shows results obtained with three other genes. Mix1 (*Xmix1*) is a homeobox gene described by Rosa (20) as being

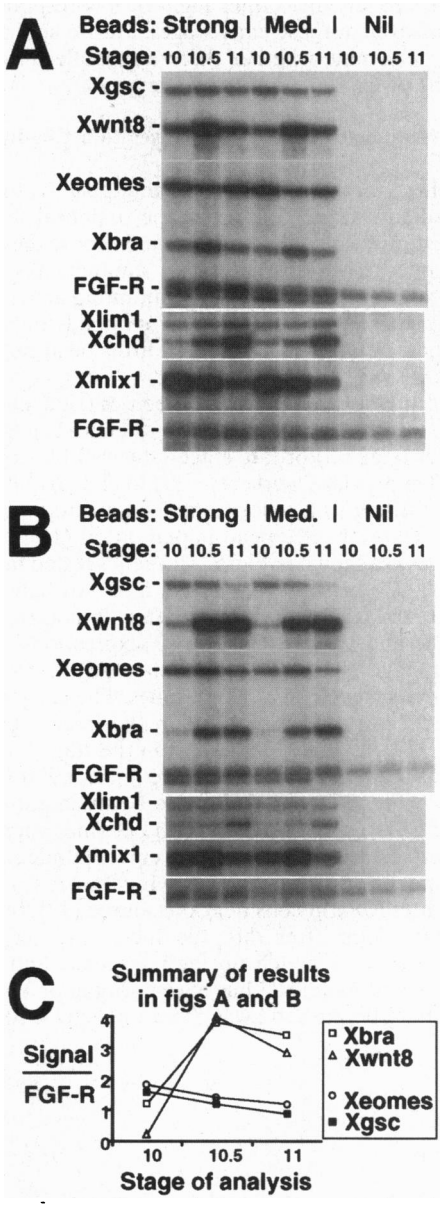

FIG. 5. Temporal sequence of gene response. RNase protection analyses of constructs containing strong (32 nM), medium (16 nM), or nil activin beads. Constructs were prepared with animal caps at stage 8 (*A*) or stage 9 (*B*) and frozen at the stages shown. Stage 10 is reached 2 hr after stage 9 and 4 hr after stage 8. Stages 10.5 and 11 are reached 1.5 and 3 hr, respectively, after stage 10. FGF-R, FGF-receptor as a loading control; see text for the genes assayed. Each track is an analysis of three constructs. *C* includes the results of both *A* and *B* after quantitation by PhosphorImager (Molecular Dynamics) analysis. The values for *Xgsc* and *Xbra* have been increased by 2.1 and 3.1, respectively, for more convenient visual comparison.

expressed in the endoderm and vegetal mesoderm at stages 9 and 10. *Xpo* is expressed in the posterior mesoderm during gastrula and neurula stages (21). Finally *Xeomesodermin* (*Xeomes*) is a gene newly discovered in this laboratory and expressed in the mesoderm at stage 9 and 10 (K.R., N. Garrett, A.M., and J.B.G., unpublished data). The results with *Xmix1* and *Xeomes* can be tested at stage 10 and therefore compared directly with *Xgsc* and *Xbra* expression already known to us (Fig. 3 *A* and *B*). Clearly both *Xmix1* and *Xeomes* respond to high activin concentrations, much as *Xgsc* does (Fig. 3 *C–F*). On the other hand, the expression of *Xpo* (Fig. 3*G*) is similar to that of *Xbra*, even though the late appearance of *Xpo*, at stage 11, makes it hard to be sure whether *Xpo* exactly coincides with *Xbra*. In conclusion, we find, so far, that genes seem to fall broadly into either high- or low-response categories when assayed at these early stages. Future analyses will be needed to determine whether there are subtle differences in the patterns of expression of these genes.

Temporal Sequence of Gene Responses to a Gradient

To unravel the means by which different genes respond to an activin gradient, we need to know the temporal sequence of their expressions. This information could expose the possibility that one gene activates or represses another. To determine this, we have prepared conjugates containing activin beads of different strengths; conjugates were stopped for analysis at various times, but in all cases, >2 hr after bead implantation (see above).

RNase protection analysis has been carried out on four series of conjugates: medium- or high-strength beads in animal caps of mid- (stage 8) or late-stage (stage 9) blastulae. It has been found in previous work, referred to above, that genes are induced according to developmental stage and not according to the time elapsed since bead implantation (13).

The results are shown in Fig. 5. The genes tested fall into two classes. *Xgsc, Xmix1*, and *Xeomes* are most strongly expressed at stage 10, and less strongly thereafter, in contrast to *Xbra, Xwnt8* (22), and *Xchd* (23), whose expressions are much stronger at stage 10.5 and 11 than at stage 10. *Xlim1* (24) is about evenly expressed at all these times. The early expression of *Xgsc* and *Xeomes*, compared with the later expression of *Xwnt8* and *Xbra*, is particularly clear in the stage 9 conjugates (Fig. 5*B*). When all four series are grouped together for comparison (Fig. 5*C*), the difference between early and late patterns of gene activation by activin becomes very clear.

These new results can be related to current understanding of our experimental system in the following way. A strong activin signal activates *Xbra* in cells near the source (13), but only for a short time. Soon after this, the increasing activin signal causes these cells to switch up their response and therefore activate *Xgsc* and *Xeomes*. Thus a weak ripple of *Xbra* expression appears to be pushed away from the activin source until a stable gradient is achieved. We suggest that RNase protection detects *Xbra* RNA only when a sufficient amount has accumulated. This will occur when the *Xbra* ripple has stopped moving and after the time when *Xgsc* and *Xeomes* transcripts have already been seen. These findings encourage future work to ask whether overexpression of *Xgsc* and *Xeomes* induces or represses the expression of other genes, especially *Xwnt8* and *Xbra*. It is interesting that these two early response genes are mainly dorsal, in contrast to the two later expressing genes, which are ventral or lateral in their normal mesodermal expression.

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