Analysis of competence and of Brachyury autoinduction by use of hormone-inducible Xbra

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SUMMARY

Analysis of gene function in Xenopus development frequently involves over-expression experiments, in which RNA encoding the protein of interest is microinjected into the early embryo. By taking advantage of the fate map of Xenopus, it is possible to direct expression of the protein to particular regions of the embryo, but it has not been possible to exert control over the timing of expression; the protein is translated immediately after injection. To overcome this problem in our analysis of the role of Brachyury in Xenopus development, we have, like Kolm and Sive (1995; Dev. Biol. 171, 267-272), explored the use of hormone-inducible constructs. Animal pole regions derived from embryos expressing a fusion protein (Xbra-GR) in which the Xbra open reading frame is fused to the ligand-binding domain of the human glucocorticoid receptor develop as atypical epidermis, presumably because Xbra is sequestered by the heat-shock apparatus of the cell.

Addition of dexamethasone, which binds to the glucocorticoid receptor and releases Xbra, causes formation of mesoderm. We have used this approach to investigate the competence of animal pole explants to respond to Xbra-GR, and have found that competence persists until late gastrula stages, even though by this time animal caps have lost the ability to respond to mesoderm-inducing factors such as activin and FGF. In a second series of experiments, we demonstrate that Xbra is capable of inducing its own expression, but that this auto-induction requires intercellular signals and FGF signalling. Finally, we suggest that the use of inducible constructs may assist in the search for target genes of Brachyury.

Key words: Xenopus, mesoderm, Brachyury, steroid receptor, competence, target gene, FGF

INTRODUCTION

Analysis of gene function in Xenopus laevis is frequently carried out by means of over-expression experiments, in which RNA encoding the protein of interest is injected into the early embryo. It is possible, in this way, to exert control over which cells express the protein by taking advantage of the accurate fate map of Xenopus (Dale and Slack, 1987; Moody, 1987). One difficulty with this approach, however, is that injected RNA is translated immediately (Snape and Smith, 1996) and this may lead to difficulties in interpreting the results of experiments of this sort. For example, expression of Xwnt-8 by RNA injection mimics the dorsalising action of the Spemann organizer, while expression at the early gastrula stage, under the control of the cytoskeletal actin promoter, causes formation of ventral mesoderm (Christian and Moon, 1993). In order to exert control over when exogenous proteins become functional, and thereby overcome this problem, Kolm and Sive (1995) used techniques borrowed from work in cell culture (Hollenberg et al., 1993) and microinjected Xenopus embryos with RNA encoding a fusion protein in which MyoD is fused to the hormone-binding domain of the glucocorticoid receptor. In the absence of hormone, the MyoD fusion protein was sequestered by the heat-shock apparatus of the cell, and the embryo developed normally. Addition of dexamethasone, however, liberated the fusion protein and caused the formation of ectopic muscle.

Mesoderm induction in amphibian development is believed to involve members of the FGF and TGF-β families (see Slack, 1994). One of the direct targets of these mesoderm-inducing factors is Xenopus Brachyury (Xbra) (Smith et al., 1991), a gene expressed throughout the presumptive mesoderm at the early gastrula stage, with expression later becoming restricted to notochord and posterior tissues (Herrmann et al., 1990; Wilkinson et al., 1990; Smith et al., 1991; Schulte-Merker et al., 1992). Ectopic expression of Xbra in animal pole explants is sufficient to induce mesoderm formation in a concentration-dependent manner. Low concentrations cause the formation of ventral mesoderm, including somitomere and mesenchyme, while higher concentrations induce dorsal mesoderm including somitic muscle (Cunliffe and Smith, 1992, 1994). Expression of Xbra alone cannot induce notochord, but co-expression with either noggin or Pintallavis, each of which is expressed in dorsal mesoderm, does cause formation of notochord (Cunliffe and Smith, 1994; O’Reilly et al., 1995).

Brachyury encodes a nuclear DNA-binding protein (Kispert and Herrmann, 1993; Cunliffe and Smith, 1994) and functions as a transcription activator (Kispert et al., 1995; Conlon et al., 1996). The mechanism by which Brachyury induces mesoderm is, however, poorly understood, and nothing is known about
potential target genes. In this paper, we begin to investigate these questions using an inducible version of Xbra in which the protein is fused to the hormone-binding domain of human glucocorticoid receptor, thus generating Xbra-GR. The activity of Xbra-GR is tightly regulated by dexamethasone (DEX) both in animal pole explants and in whole embryos. We first use this construct to study the competence of animal pole tissue to form mesoderm in response to Xbra. Our results show that competence persists well into gastrulation, when animal caps can no longer respond to activin or FGF. The second group of experiments addresses the question of target genes, where we investigate the mechanism of Brachyury autoinduction. DEX-induced transactivation of the endogenous Xbra gene in animal pole explants expressing Xbra-GR is inhibited by co-expression of a truncated FGF receptor, and does not occur in dissociated animal pole blastomeres. These results indicate that the direct interaction of Xbra with its own promoter is not sufficient to drive transcription, and that Xbra autoinduction requires intercellular signals, presumably through secretion of a member of the FGF family. In future work we hope to use this system to isolate genes that are direct targets of Xbra.

MATERIALS AND METHODS

Construction of inducible versions of Brachyury

The Xbra-GR construct was generated by fusing the coding region (amino acids 1-432) of pSP64T-Xbra (Cunliffe and Smith, 1992) to a fragment encoding the hormone-binding domain (amino acids 512-777) of the human glucocorticoid receptor (hGR) derived from plasmid p64T-MyoD-GR (Hollenberg et al., 1993) by in vitro mutagenesis and PCR. The GR construct was generated by addition of an initiation methionine at the amino-terminus of the hormone-binding domain of hGR using PCR. Fragments encoding Xbra-GR and GR were cloned into the vector pSP64T (Krieg and Melton, 1984) and designated pSP64T-Xbra-GR and pSP64T-GR. Two copies of the HA epitope (YPYDVPDYA) (Field et al., 1988), which can be recognized by the monoclonal antibody 12CA5 (Boehringer Mannheim), were introduced at the carboxy-terminus of these proteins to generate pSP64T-Xbra-GR, pSP64T-Xbra-GR-HA and pSP64T-GR-HA.

An Xbra-ERT™ construct was generated by replacing the region encoding the hormone-binding domain of Xbra-GR with a fragment encoding a modified ligand-binging domain of the murine estrogen receptor. This domain is unable to bind estrogen but has high affinity for the synthetic ligand 4-hydroxytamoxifen (4-OHT) (Littlewood et al., 1995). A preliminary series of experiments revealed that this construct induced ectopic mesoderm even in the absence of 4-OHT.

Xenopus embryos, microinjection, dissection and mesoderm-inducing factors

Xenopus embryos were obtained by in vitro fertilization (Smith and Slack, 1983). They were maintained in 10% Normal Amphibian Medium (NAM; Slack, 1984) and staged according to Nieuwkoop and Faber (1967). Xenopus embryos at the one- to two-cell stage and the 32-cell stage were injected with RNA dissolved in 10 nl and 1 nl water, respectively, as described by Smith (1993). For animal cap assays, embryos were dissected and cultured in 75% NAM or 75% NAM containing 0.1% bovine serum albumin when mesoderm-inducing factors were included in the culture medium. Xenopus bFGF was prepared using an expression plasmid provided by David Kimelman and Marc Kirschner. A crude preparation of recombinant human activin A was prepared from the conditioned medium of COS cells transfected with a human inhibin βA cDNA. The cells were a gift from Dr Gordon Wong (Genetics Institute Inc, Massachusetts). A unit of activin activity is defined by Cooke et al. (1987). Dexamethasone (Sigma) was dissolved in ethanol at 2 mM and then diluted to a final concentration of 1 μM in 75% NAM for animal caps or in 10% NAM for whole embryos.

Cell dispersal and protein synthesis inhibition experiments

Cell dispersal and protein synthesis inhibition experiments were performed as described by Smith et al. (1991), except that cycloheximide was applied continuously at a concentration of 10 μM.

In vitro transcription, in vitro translation and immunoprecipitation

All pSP64T constructs were linearised with Sall and RNA was synthesised according to Smith (1993). RNA encoding nucleolar gal was transcribed from pSP6nucGal (Smith and Harland, 1991) and RNA encoding the dominant negative FGF receptor, XFD, was transcribed from pXFD/Ass (Amaya et al., 1991). In vitro translations of HA-tagged constructs were carried out in rabbit reticuloocyte lysate in the presence of [35S]methionine, using 200 ng of synthetic RNA, according to the manufacturer’s recommendations (Promega). Immunoprecipitation was performed as described by Cunliffe and Smith (1994) with the use of anti-HA monoclonal antibody 12CA5.

Western blotting

Extraction of protein from animal caps was as described by Cunliffe and Smith (1994). Protein extracted from the equivalent of two animal caps was analysed on a 12% acrylamide gel by SDS-PAGE and then blotted to a PVDF membrane (Immobilon, Millipore) by wet electrophoretic transfer. The membrane was reacted with anti-HA monoclonal antibody 12CA5 and subsequently with anti-mouse IgG conjugated with alkaline phosphatase (Sigma) followed by detection with NBT and BCIP.

Histology, immunocytochemistry and β-gal staining

For histological analysis, specimens were fixed, sectioned and stained as described by Smith (1993). Whole-mount and tissue section immunocytochemistry with monoclonal antibodies MZ15 (Smith and Watt, 1985) or 12/101 (Kintner and Brockes, 1984), specific for notochond and muscle, respectively, was performed as described by Smith (1993) and Cunliffe and Smith (1994). β-gal staining was carried out essentially as described by Whiting et al. (1991). Embryos were fixed in 1% paraformaldehyde, 0.2% glutaraldehyde, 2 mM MgCl2, 5 mM EGTA, and 0.02% NP-40 in PBS at 4°C for 1 hour. They were then washed in PBS containing 0.02% NP-40 and stained in 1 mg/ml X-gal, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2, 0.01% sodium deoxycholate, and 0.02% NP-40 at room temperature.

Whole-mount in situ hybridization

The protocol of Harland (1991) was used with minor modifications, including the use of BM purple as a substrate and without treatment of RNAaseA. An unhybridised Xbra probe was prepared by linearising pXT1 (Smith et al., 1991) with StuI, followed by transcribing with T7 RNA polymerase in the presence of digoxigenin (DIG)-labelled UTP. The probe corresponds to the 3'-untranslated region of Xbra to avoid cross-hybridisation with RNA encoding Xbra-GR.

RNA isolation and RNAase protection assays

RNA was extracted from animal caps by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) followed by lithium precipitation. RNAase protection analysis was performed essentially as described by Jones et al. (1992). RNAase digestion was done with RNAase T1 alone for all probes. Samples were analysed with probes specific for EF-1α (Sargent and Bennett, 1990), ornithine decarboxylase (ODC) (Isaacs et al., 1992), cardiac actin (Mohun et al., 1984), Xbra (Smith et al., 1991), Xhox3 (Ruiz i Altaba

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Fig. 1. Construction of Xbra-GR. The hormone binding domain of the human glucocorticoid receptor (hGR) amino acids 512-777 was fused to the carboxy-terminus of Xbra (1-432) to generate Xbra-GR. This fusion replaces the stop codon of Xbra with two amino acids (DL) generated by the insertion of a BglII site (see Materials and methods). The DNA-binding and activation domains of Xbra are shown by shaded and hatched boxes, respectively (see Kispert and Herrmann, 1993; Conlon et al., 1996). The hormone-binding domain of hGR is shown by a solid box.

and Melton, 1989) and CAT. For CAT, pTriCAT (Ambion) was transcribed with T7 RNA polymerase, thus generating a probe of 241 nucleotides. The protected fragment is 150 nucleotides long.

RESULTS

Xbra-GR induces mesoderm in animal cap explants in a hormone-dependent manner

Following Kolm and Sive (1995), we have tested a construct in which Xbra is fused to the ligand-binding domain of the glucocorticoid receptor (Xbra-GR; see Fig. 1). Xbra functions as a transcription activator (Kispert et al., 1995; Conlon et al., 1996) and we first asked whether Xbra-GR is capable of activating expression of a target gene in animal cap explants in a hormone-dependent manner. *Xenopus* embryos were injected with RNA encoding Xbra-GR along with a chloramphenicol acetyl transferase (CAT) reporter plasmid which contains two copies of the palindromic Brachyury binding site (Kispert and Herrmann, 1993) upstream of a thymidine kinase minimal promoter (Fig. 2A; Conlon et al., 1996). Animal caps derived from these embryos were cultured for 3 hours in the presence or absence of DEX and then assayed for expression of CAT mRNA. DEX caused a marked stimulation of CAT expression (Fig. 2B), indicating that Xbra-GR activates a putative target gene in animal cap explants.

To investigate whether Xbra-GR activates mesoderm in animal caps in a DEX-dependent manner, animal caps derived from embryos injected with RNA encoding Xbra-GR were cultured in the presence or absence of DEX and subjected to morphological and histological analyses at stage 42 (Fig. 3).

In control experiments, addition of DEX to animal caps derived from uninjected embryos, or from embryos injected with RNA encoding GR alone, did not form mesoderm (Fig. 3A-D, M-P). Similarly, addition of DEX to animal caps derived from embryos injected with 400 pg RNA encoding wild-type Xbra neither enhanced nor repressed mesoderm formation (Fig. 3E-H). However, injection of the same amount of RNA encoding Xbra-GR did not cause mesoderm formation unless DEX was added to the medium (Fig. 3I-L). Interestingly, DEX treatment of animal caps expressing Xbra-GR reproducibly caused the formation of large masses of muscle (Fig. 3K,L; see also Fig. 5F), whereas wild-type Xbra usually induced ventral mesoderm (Fig. 3E-H).

The mesoderm-inducing activity of Xbra-GR was also assessed by analysing the expression of various mesodermal markers (Fig. 4). Addition of DEX to animal pole regions derived from embryos injected with RNA encoding Xbra-GR induced expression of *Xho3*, a ventroposterior marker, but not *goosecoid*, a dorsoanterior marker. In these respects the effects of Xbra-GR resemble those of wild-type Xbra (Fig. 4 and Cunliffe and Smith, 1992). Consistent with the histological data described above, DEX treatment of animal pole regions derived from embryos injected with RNA encoding Xbra-GR caused stronger expression of muscle-specific actin than did the same amount of RNA encoding wild-type Xbra.

Previous work has indicated that different concentrations of

Table 1. Morphology of animal caps derived from embryos injected with increasing amounts of *Xbra-GR* RNA

<table>
<thead>
<tr>
<th>Xbra-GR RNA (pg)</th>
<th>Uninduced vesicles</th>
<th>Muscle</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>Dex (-)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>14</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>400</td>
<td>10</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Dex* (+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>1</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>400</td>
<td>-</td>
<td>-</td>
<td>15</td>
</tr>
</tbody>
</table>

*The concentration used was 10^{-6} M.*
Xbra induce different types of mesoderm: low concentrations induce ventral mesoderm containing mesothelium and mesenchyme, while higher concentrations induce muscle (O’Reilly et al., 1995). This raises the possibility that Xbra-GR induces large amounts of muscle because it is a more efficient inducer of mesoderm than wild-type Xbra. To investigate this, different amounts of Xbra-GR RNA were injected into fertilized Xenopus eggs and animal caps derived from the resulting embryos were exposed to DEX. Animal caps derived from embryos injected with 25 pg Xbra-GR RNA formed ventral mesodermal vesicles containing mesothelium and mesenchyme, similar to those formed in response to 400 pg of Xbra RNA (Table 1; Fig 5B). Injection of 400 pg Xbra-GR RNA caused most explants to form large masses of muscle (Table 1; Fig. 5C,F). Two explants out of 45 even contained a small patch of notochord-like tissue (data not shown). RNAase protection analysis of similar explants confirmed that increasing amounts of Xbra-GR RNA elicit the formation of increasing amounts of muscle (data not shown). Together, these results indicate that Xbra-GR has stronger mesoderm-inducing activity in animal cap explants than wild-type Xbra (see Discussion).

Over-expression of Xbra-GR in intact Xenopus embryos

Previous work has shown that microinjection of Xbra RNA into fertilized Xenopus eggs results in severe perturbation of gastrulation, due to the formation of ectopic mesoderm in the animal hemisphere of the embryo (Cunliffe and Smith, 1992). These experiments do not reveal, however, when Xbra exerts its effects. To investigate this question we have injected animal pole blastomeres of the 32-cell stage Xenopus embryo with 10 pg Xbra-GR RNA and then treated the embryo at stage 8, 10 or 12 with DEX. Addition of DEX at stage 10 causes embryos to form tail-like protrusions (Fig. 6B,C). When the protrusions are present in the head, the embryos lack eyes (Fig. 6C) and when they occur near the tail, they contain fin-like structures (Fig. 6D). Exposure to DEX at stages 8 or 12 was less efficient in this assay than treatment at stage 10, the normal time of onset of Xbra expression.
Increasing amounts of RNA encoding Xbra-GR induce different types of mesoderm. Histological (A-C) and immunohistochemical (D-F) analyses of animal caps derived from embryos injected with increasing amounts of RNA encoding Xbra-GR. Animal caps were dissected at stage 8, treated with $10^{-6}$ M DEX, and cultured to stage 42 when they were fixed and sectioned at 7 μm for histological analysis or at 10 μm for immunohistochemical analysis. For histological analysis (A-C), the sections were stained by the Feulgen technique and with Light Green and Orange G. For immunohistochemical analysis (D-F), the sections were stained with 12/101 monoclonal antibody for muscle. (A,D) Untreated caps treated with DEX form atypical epidermis. (B,E) DEX-treated animal caps derived from embryos injected with 25 pg of RNA encoding Xbra-GR form ventral mesodermal vesicles, which contain mesothelium and mesenchyme. (C,F) DEX-treated animal caps derived from embryos injected with 400 pg of RNA encoding Xbra-GR form clumps of muscle cells. This muscle tissue is typical of caps derived from embryos injected with 100 pg of RNA encoding Xbra-GR or more.

Effects of injected RNA encoding Xbra-GR in whole embryos. (A-D) One animal pole blastomere (tier A) of embryos at the 32-cell stage received injections of RNA encoding Xbra-GR (10 pg). Embryos were left untreated (A) or exposed to $10^{-6}$ M DEX at stage 10 (B-D). They were allowed to develop to stage 34. DEX-treated embryos typically possess tail-like protrusions (B,C). When protrusions are present in the head, embryos lack eyes (C) and when they occur near the tail, they contain fin-like structures (D). The tail-like protrusions are shown by arrows. Treatment with DEX at stage 10 was more effective than that at stage 8 or 12. (E-H) Embryos were injected in one blastomere of tier A (E,F) or tier C (G,H) at the 32-cell stage with 10 pg of RNA encoding Xbra-GR together with nucβ-gal RNA. The embryos were treated with $10^{-6}$ M DEX at stage 10 (F,H) or were untreated (E,G), fixed at stage 34 and stained with X-gal. Embryos injected in tier A and treated with DEX possess tail-like protrusions (see Table 2). When Xbra-GR and nucβ-gal RNAs were injected into a tier A blastomere in the absence of DEX, labelled cells were found in the surface ectoderm (E) or in neural tissues, but addition of DEX at stage 10 changed the fate of these cells causing them to form tail-like protrusions (98% of cases; n=67) (F). In contrast, following injection into a tier C blastomere, labelled cells were observed in the notochord or in the paraaxial cells (G) even after treatment with DEX (H). (I-L) Whole-mount immunostaining analysis of embryos injected with 10 pg of RNA encoding Xbra-GR into a tier A blastomere at the 32-cell stage. The embryos were left untreated (I,K) or treated with $10^{-6}$ M DEX at stage 10 (J,L), fixed at stage 34 and stained with MZ15 monoclonal antibody for notochord (I,J) or with 12/101 monoclonal antibody for muscle (K,L). Tail-like protrusions are negative for notochord (J), while they are positive for muscle (arrow in L).
Consistent with this, injection of wild-type Xbra RNA has less activity than Xbra-GR, even when 400 pg RNA is injected (Table 2).

We next asked whether the ectopic tail-like protrusions are produced by Xbra-GR-expressing cells themselves and whether they can form from equatorial blastomeres as well as animal pole cells. In this experiment, tier A or tier C (equatorial) blastomeres were injected with Xbra-GR RNA together with β-galactosidase RNA. The results are summarized in Table 2. In the absence of DEX, injection into a tier A blastomere results in labelled cells being found in the surface ectoderm (Fig. 6E) or in neural tissues, whereas addition of DEX at stage 10 causes the labelled cells to populate a tail-like protrusion (Fig. 6F). Some of the ectopic tail-like tissue was recruited from uninjected cells. In contrast, addition of DEX to embryos receiving injections into tier C had little effect; labelled cells were restricted to notochord and paraxial mesodermal tissues (compare Fig. 6G and H). These results confirm that ectopic expression of Xbra can change the fate of injected cells, but indicate that it is more difficult to change the fate of equatorial cells, which express the endogenous gene to high levels.

Tucker and Slack (1995) state that a bone fide tail bud consists of notochord, somite and neural tissues. The tail-like protrusions induced by injection of RNA encoding Xbra-GR were therefore analysed by whole-mount immunohistochemistry using antibodies specific for notochord and for somitic muscle. Notochord-specific staining was not detected in Xbra-GR-induced tail-like protrusions (Fig. 6J), except in one case where the ectopic tail formed close to the host notochord (not shown). By contrast, well-organized somitic muscle was consistently observed (Fig. 6L). Thus, expression of Xbra alone is sufficient to cause formation of a tail-like protrusion, but cannot induce a bone fide tail.

### Competence to respond to Xbra persists during gastrulation

The competence of animal pole explants to respond to mesoderm-inducing factors such as activin or FGF is lost by the early gastrula stage (Green et al., 1990). One way this might occur is through loss of receptors for inducing factors, as suggested by Gillespie et al. (1989). Alternatively, the signal transduction pathways for inducing factors might remain intact, but cells could become refractory to the effects of genes such as Xbra that are activated by these pathways. To investigate this question, we have used our hormone-inducible construct to examine the competence to respond to Xbra at different developmental stages.

Preliminary experiments examined the stability of the Xbra-GR protein in Xenopus embryos. Immunoprecipitation analysis of in vitro translated protein derived from Xbra-HA, Xbra-GR-HA and GR-HA, all of which include repeated nonapeptides from the influenza hemagglutinin protein (HA), using anti-HA monoclonal antibody 12CA5 (see Materials and methods). Immunoprecipitates were analysed by 12% acrylamide gel electrophoresis followed by fluorography. Positions of molecular mass markers (×10^3) are indicated. (B) Western blotting analysis of Xbra-GR protein levels in Xenopus embryos. Animal caps from embryos injected with 400 pg of RNA encoding Xbra-GR-HA were dissected at stage 8, treated with 10^{-6} M DEX where appropriate and collected at the indicated stages. Two cap equivalents of protein were analysed by western blotting using the anti-HA antibody after 12% acrylamide gel electrophoresis. Positions of molecular mass markers (×10^3) are indicated. The band corresponding to the protein of Xbra-GR-HA is indicated by an arrowhead. Note that Xbra-GR-HA protein levels are stable in the absence of DEX but fall rapidly after its addition.

### Table 2. Targeted injection of Xbra-GR RNA

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Injection</th>
<th>Tail-like protrusions (%)</th>
<th>Tiny protrusions (%)</th>
<th>Gastrulation defect (%)</th>
<th>Unaffected (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>Xbra</td>
<td>17</td>
<td>11</td>
<td>2</td>
<td>70</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>Xbra-GR</td>
<td>61</td>
<td>16</td>
<td>1</td>
<td>98</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>Dex (+)</td>
<td>61</td>
<td>16</td>
<td>1</td>
<td>22</td>
<td>83</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>Xbra-GR</td>
<td>7</td>
<td>93</td>
<td>15</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tier A</td>
<td>80</td>
<td>13</td>
<td>7</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Tier C</td>
<td>47</td>
<td>33</td>
<td>20</td>
<td>15</td>
<td>7</td>
</tr>
</tbody>
</table>

Embryos were injected in one blastomere (animal tier or marginal tier) at 32 cell stage with 400 pg of Xbra RNA or 10 pg of Xbra-GR RNA. The embryos were scored by morphology at stage 34. *Embryos were treated with Dex at stage 10.

Fig. 7. Stability of Xbra-GR protein in Xenopus embryos.
(A) Immunoprecipitation analysis of in vitro translated protein derived from Xbra-HA, Xbra-GR-HA and GR-HA, all of which include repeated nonapeptides from the influenza hemagglutinin protein (HA), using anti-HA monoclonal antibody 12CA5 (see Materials and methods). Immunoprecipitates were analysed by 12% acrylamide gel electrophoresis followed by fluorography. Positions of molecular mass markers (×10^3) are indicated. (B) Western blotting analysis of Xbra-GR protein levels in Xenopus embryos. Animal caps from embryos injected with 400 pg of RNA encoding Xbra-GR-HA were dissected at stage 8, treated with 10^{-6} M DEX where appropriate and collected at the indicated stages. Two cap equivalents of protein were analysed by western blotting using the anti-HA antibody after 12% acrylamide gel electrophoresis. Positions of molecular mass markers (×10^3) are indicated. The band corresponding to the protein of Xbra-GR-HA is indicated by an arrowhead. Note that Xbra-GR-HA protein levels are stable in the absence of DEX but fall rapidly after its addition.

The competence of animal pole explants to respond to mesoderm-inducing factors such as activin or FGF is lost by the early gastrula stage (Green et al., 1990). One way this might occur is through loss of receptors for inducing factors, as suggested by Gillespie et al. (1989). Alternatively, the signal transduction pathways for inducing factors might remain intact, but cells could become refractory to the effects of genes such as Xbra that are activated by these pathways. To investigate this question, we have used our hormone-inducible construct to examine the competence to respond to Xbra at different developmental stages.

Preliminary experiments examined the stability of the Xbra-GR protein in the Xenopus embryo. Influenza hemagglutinin protein (HA)-tagged Xbra constructs can readily be detected with the anti-HA monoclonal antibody 12CA5 (Fig. 7A). RNA encoding Xbra-GR-HA was therefore injected into Xenopus embryos at the one-cell stage and animal pole explants were dissected at stage 8. Half were treated with DEX and they were then cultured and analysed by western blotting at stage 10, 12 or 14. In the absence of DEX, levels of Xbra-GR-HA remain constant at least to stage 14 (Fig. 7B). Addition of DEX, however, caused rapid loss of Xbra-GR-HA (Fig. 7B). Together, these results suggest that Xbra-GR-HA, like MyoD-GR (Kolm and Sive, 1995) is remarkably stable. This is likely to be due to association with cytoplasmic heat-shock protein 90, because addition of DEX causes rapid degradation. Consistent with this suggestion, Xbra-HA is less stable than the...
The ability of animal cap explants to respond to Xbra-GR, activin or FGF was assessed by morphological criteria at stage 20 (data not shown) and expression of cardiac actin at stage 23 (Fig. 8A). By both criteria, competence to respond to bFGF was lost by stage 10 and responsiveness to activin was lost by stage 11. In contrast, explants derived from embryos injected with RNA encoding Xbra-GR were able to respond to DEX even at stage 11, and further experiments revealed that DEX was still capable of inducing cardiac actin expression at stage 12 (Fig. 8B). By stage 13, activation of Xbra-GR did not induce cardiac actin, but ventral mesoderm was formed (data not shown). These experiments indicate that the ability to respond to Xbra persists longer than the ability to respond to either FGF or activin.

It is possible that low levels of DEX-independent Xbra activity during blastula and early gastrula stages cause competence to respond to mesoderm induction to be extended. To address this point, competence to respond to FGF or activin was assessed in the presence of Xbra-GR but in the absence of DEX. In these experiments, as in the absence of Xbra-GR, competence to respond to FGF and activin was lost at stages 10 and 11, respectively, while competence to respond to Xbra-GR persisted beyond stage 11 (data not shown).

**Indirect autoinduction of Brachyury**

Experiments in mouse, zebrafish and Xenopus embryos indicate that maintenance of Brachyury expression requires Brachyury function (Herrmann, 1991; Schulte-Merker et al., 1994; Conlon et al., 1996). Consistent with this, Rao has demonstrated that Xbra can activate its own expression in Xenopus animal caps (Rao, 1994), and we have confirmed this using hormone-inducible Xbra both in animal caps (Fig. 9A) and in whole embryos (Fig. 9B,C).

These results raise the possibility that one target of Brachyury, a transcription activator, is Brachyury itself, and we have used Xbra-GR to investigate whether interaction of Xbra with its own promoter is sufficient to drive transcription. In the first experiment we compared the time-course of Xbra induction by FGF (which activates Xbra in an immediate-early fashion) and by Xbra itself (Fig. 10A). Induction of Xbra in animal pole explants was first detectable 2 hours after exposure to FGF, while in animal caps derived from embryos injected with RNA encoding Xbra-GR, Xbra transcripts were not observed until 3 hours after addition of DEX.

To assess whether Xbra autoinduction requires protein synthesis, animal pole explants derived from embryos injected with Xbra-GR RNA were treated with DEX in the continuous presence or absence of cycloheximide. DEX-induced Xbra expression was dramatically reduced by cycloheximide, but induction of Xbra by FGF was also slightly inhibited, presumably due to inhibition of cell division of animal pole blastomeres by cycloheximide (data not shown). As an alternative test for the direct action of Xbra, we therefore asked whether Xbra autoinduction could occur in dissociated animal pole blastomeres (Smith et al., 1991). DEX-induced Xbra expression was not observed in dissociated animal cap cells, while Xbra expression was induced under the same conditions by bFGF (Fig. 10B). Together with the time-course experiment, these results suggest that Xbra autoinduction requires protein synthesis and intercellular signals.

Several lines of evidence suggest that Xbra and eFGF are involved in an autoregulatory loop in which Xbra induces eFGF expression, and eFGF is required for maintenance of Xbra expression (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). To examine the possibility that autoinduction of Xbra involves FGF signalling, RNA encoding a truncated FGF receptor, XFD, was injected into Xenopus embryos along with Xbra-GR RNA. In confirmation of previous work, XFD blocked mesoderm induction by both FGF and by Xbra (Fig. 11A), and additional experiments demonstrated that Xbra autoinduction also requires FGF signalling (Fig. 11B). Analysis of Xbra expression at different times after addition of DEX reveals that the truncated FGF receptor blocks the initial induction of Xbra, and that the lack of expression seen in Fig. 11B is not due to inhibition of maintenance of expression (data not shown).
DISCUSSION

Hormone-dependent Xbra

In this paper, we demonstrate that a hormone-inducible version of Xbra, Xbra-GR, is functional both in animal caps and in whole embryos. Like wild-type Xbra, Xbra-GR induces different mesodermal cell types at different concentrations, although we note that less Xbra-GR RNA is required than wild-type RNA for strong induction of muscle. This is likely to be due to the greater stability of Xbra-GR protein in the embryo, as is also the case for a MyoD-GR fusion protein (Kolm and Sive, 1995). This greater stability is likely to be due to association of Xbra-GR with the cytoplasmic protein hsp90, an association which blocks the activity of Xbra until DEX is added (see Smith and Toft, 1993). It is also possible, however, that the greater activity of the fusion protein is due to the TAFII activity of the ligand-binding domain of the glucocorticoid receptor (see Mattioni et al., 1994) or to enhanced dimerization of the Xbra-GR protein caused by the GR moiety (see Fawell et al., 1990).

We have used Xbra-GR to study the duration of competence to respond to Xbra and to ask whether Xbra activates its own transcription directly, by acting on its own promoter, or indirectly. The approach we describe will be applicable to other transcription factors, and may help in the identification of their downstream targets.

Duration of competence to respond to Xbra

A particular advantage of using hormone-inducible constructs like Xbra-GR is that it allows control of the effective timing of expression of genes such as Xbra, MyoD (Kolm and Sive, 1995) and, most recently, Otx2 (Gammill and Sive, 1997). Using this approach we have demonstrated that induction of tail-like structures in whole embryos is most efficient if expression occurs at stage 10, and we have shown that the competence of animal pole explants to respond to Xbra persists during gastrulation (Fig. 8), even when the response to mesoderm inducing factors, such as FGF and activin, has been lost (Green et al., 1990).

This latter result impinges on the question of what controls the nature of the response that cells make to inducing factors. FGF has recently been shown to have two separable effects on animal pole explants, depending on the time during development that it is applied. Before the onset of gastrulation it functions as a mesoderm-inducing factor, whereas if applied after the onset of gastrulation it induces neural tissue (Kengaku
Animal caps were dissected at stage 8, treated as appropriate with negative FGF receptor (XFD) and/or 100 pg of RNA encoding Xbra. One-cell stage were injected with 1 ng of RNA encoding a dominant negative mutant of the FGF receptor disrupts mesoderm formation in response to Xbra requires FGF signalling. Embryos at the one-cell stage were injected with 25 pg of RNA encoding a dominant negative FGF receptor (XFD) and/or 100 pg of RNA encoding Xbra. Animal caps were dissected at stage 8, treated as appropriate with \(10^{-6}\) M DEX and cultured to the equivalent of stage 10.5, when they were analysed for expression of cardiac actin gene by RNAase protection. Inhibition of FGF signalling blocks induction of mesoderm by Xbra-GR. Injection of cardiac actin gene expression by bFGF (50 ng/ml) in control animal caps was abolished by injection of RNA encoding XFD (1 ng) under the same conditions. (B) Xbra autoinduction requires FGF signalling. Embryos at the one-cell stage were injected with 25 pg of RNA encoding Xbra-GR and/or 1 ng of RNA encoding XFD. Animal caps were dissected at stage 8, treated as appropriate with \(10^{-6}\) M DEX and cultured to the equivalent of stage 10.5, when they were analysed for expression of Xbra by RNAase protection. Inhibition of FGF signalling prevented autoinduction of Xbra. Expression of Xbra induced by bFGF treatment (100 ng/ml) in control caps was abolished by injection of RNA encoding XFD (1 ng) under the same conditions. ODC serves as a control for loading in samples in these experiments.

**Downstream targets of Brachury**

Mutant analyses in mouse and zebrafish embryos (Herrmann et al., 1990; Halpern et al., 1993; Schulte-Merker et al., 1994), and dominant-negative studies in Xenopus (Conlon et al., 1996), suggest that one function of Brachury is to activate transcription of mesoderm-specific genes which are involved in patterning posterior mesoderm and in differentiation of the notochord. No such target genes have yet been identified, but it may be possible to use Xbra-GR to search for them (see also Gammill and Sive, 1997). In this paper we demonstrate that Xbra is not able directly to activate its own transcription. Thus, induction by DEX of Xbra expression in animal caps derived from embryos injected with Xbra-GR RNA is inhibited by cycloheximide (data not shown), by cell dispersion (Fig. 10B) and by the presence of a truncated FGF receptor (Fig. 11). These results support the idea that Xbra and eFGF are components of an indirect autoregulatory loop in which Xbra induces expression of eFGF, and eFGF is required for maintenance of Xbra expression (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). They also define three criteria that any putative Xbra target must satisfy: first, that the gene must be activated by DEX in animal caps expressing Xbra-GR even in the presence of cycloheximide (see Gammill and Sive, 1997); second, that it should be activated in dispersed cells; and finally, that the gene should be activated in the presence of a truncated FGF receptor. One potential target of Xbra in Xenopus is eFGF; the expression patterns of the two genes during gastrulation are remarkably similar (Isaacs et al., 1996) and ectopic expression of Xbra induces eFGF expression in animal caps (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). We are now going on to investigate this question using inducible Xbra.

Finally, these experiments also emphasize that FGF family members play at least two roles in Xenopus mesoderm formation. Initially, they are capable of inducing mesoderm from presumptive ectodermal tissue, and their ability to do this declines around the beginning of gastrulation (Fig. 11A). Thereafter, although FGF signalling cannot induce mesoderm from naive ectodermal tissue, it is required in newly induced mesoderm for the autoinduction of Xbra (Fig. 11B), for maintenance of expression of Xbra (Isaacs et al., 1994; Schulte-Merker and Smith, 1995), and for formation of mesoderm in response to Xbra (Fig. 11A).

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**REFERENCES**


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