The Spemann organizer-expressed zinc finger gene Xegr-1 responds to the MAP kinase/Ets–SRF signal transduction pathway

Frank Panitz, Bernhard Krain1,2, Thomas Hollemann, Alfred Nordheim1,2 and Tomas Pieler3

Institut für Biochemie und Molekulare Zellbiologie, Universität Göttingen, 1Institut für Molekularbiologie, Medizinische Hochschule Hannover and 2Institut für Zellbiologie, Eberhard-Karls-Universität Tübingen, Germany

3Corresponding author
e-mail: tpieler@gwdg.de

F.Panitz and B.Krain contributed equally to this work

The transcriptional activity of a set of genes, which are all expressed in overlapping spatial and temporal patterns within the Spemann organizer of Xenopus embryos, can be modulated by peptide growth factors. We identify Xegr-1, a zinc finger protein-encoding gene, as a novel member of this group of genes. The spatial expression characteristics of Xegr-1 during gastrulation are most similar to those of Xbra. Making use of animal cap explants, analysis of the regulatory events that govern induction of Xegr-1 gene activity reveals that, in sharp contrast to transcriptional regulation of Xbra, activation of Ets–serum response factor (SRF) transcription factor complexes is required and sufficient for Xegr-1 gene expression. This finding provides the first indication for Ets–SRF complexes bound to serum response elements to be activated during gastrulation. MAP kinase signalling cascades can induce and sustain expression of both Xegr-1 and Xbra. Ectopic Xbra can induce Xegr-1 transcription by an indirect mechanism that appears to operate via primary activation of fibroblast growth factor secretion. These findings define a cascade of events that links Xbra activity to the signal-regulated control of Xegr-1 transcription in the context of early mesoderm induction in Xenopus laevis.

Keywords: egr-1/FGF/MAP kinase signalling/serum response element/Xenopus laevis

Introduction

Early embryonic differentiation is governed by two major principles, asymmetric distribution of maternal information and cell–cell communication mediated by diffusible signalling molecules. Mesoderm induction in Xenopus is a prime example for studying the molecular details of these processes (reviewed in Gurdon, 1992; Harland and Gerhart, 1997). The dorso-vegetal region of cleavage stage embryos, the so-called Nieuwkoop center (Nieuwkoop 1969, 1977), is believed to be the primary signalling source that helps to induce a group of cells to become the principal regulatory centre at gastrulation, known as the Spemann organizer (Spemann and Mangold, 1924).

A growing number of genes specifically expressed in the Spemann organizer, all encoding either transcription factors or secreted proteins, are in the process of being identified. Such genes include the homeobox gene goosecoid (gsc) (Cho et al., 1991), the T-box genes brachyury (Smith et al., 1991) and eomesodermin (Ryan et al., 1996), as well as the secreted factors noggin (Smith and Harland, 1992), Xnr3 (Smith et al., 1995) and chordin (Sasai et al., 1994). The corresponding transcription units appear to be expressed differentially within the early gastrula organizer in the animal–vegetal and superficial–deep dimensions, perhaps reflecting the subdivision of the organizer into different functional domains (Vodicka and Gerhart, 1995).

The question of how these distinct patterns of gene expression are generated at the transcriptional level has been addressed only recently; activin response elements (AREs) which share no obvious primary sequence homology have been identified in three different genes (Watabe et al., 1995; Kaufmann et al., 1996; Vize, 1996). A winged-helix transcription factor has been found to be part of an activin-inducible ARE–protein complex, which acts in concert with Smad2 and Smad4, two recently identified transforming growth factor-β (TGF-β)/activin signal transducing proteins (Baker and Harland, 1996; Chen et al., 1996, 1997; Graff et al., 1996). Additional Smad proteins have been identified as important components of the signal transduction pathway that is responsible for serine/threonine kinase receptor signalling; Smad1, for example, has a function in bone morphogenetic protein (BMP) signalling (Graff et al., 1996; Thomsen, 1996).

A second signal transduction pathway, the fibroblast growth factor (FGF)-induced MAP kinase (MAPK) pathway, has been shown to be involved in mesoderm induction and the ensuing early mesodermal patterning events (Whitman and Melton, 1992; MacNicol et al., 1993; Hartley et al., 1994; Gotoh et al., 1995; La Bonne et al., 1995; Northrop et al., 1995; Umbhauer et al., 1995; La Bonne and Whitman, 1997). One of the genes that is thought to be regulated directly by FGF via the MAPK signal transduction pathway is Xbra. A regulatory loop has been established in which Xbra activates expression of a gene encoding an FGF-type signalling molecule (XeFGF) which in turn serves to maintain expression of Xbra (Isaacs et al., 1994; Schulte-Merker and Smith, 1995; Tang et al., 1995). However, as yet no transcription factor(s) that would relay the FGF signal at the Xenopus brachyury promoter has been identified (Artinger et al., 1997; Latinkic et al., 1997).

Critical targets downstream of activated MAPK in Xenopus embryos remain to be identified. Cell culture studies have defined several transcription factors to be phosphorylated and activated by MAPKs. These include CREB, ATF2, Ets1/2 and members of the TCF subclass...
Characteristics of Xegr-1 expression in X. laevis

Fig. 1. Prediction of Xegr-1 protein sequence. (A) Schematic representation of the functional domains in the Egr-1 transcription activator (modified from Gashler and Sukhatme, 1995): 1, strong transcriptional activation domain; 2, repressor domain that interacts with a specific co-repressor protein; 3, protein domains required for nuclear transport; 4, zinc finger cluster defining the DNA-binding domain; 5, weak transcriptional activation domain. (B) Primary sequence comparison of human (Milbrandt, 1987), zebrafish (Drummond et al., 1994), and X. laevis (this study) Egr-1 proteins. The individual sequence blocks correspond to the functional domains as defined in (A). Hyphens indicate the positions of sequence identity; dots reflect deletions. The degree of sequence identity is given separately for each of the sequence blocks.

Fig. 2. Spatial distribution of Xegr-1 mRNA during Xenopus embryogenesis. Whole-mount in situ hybridization analysis first detects localized transcripts in the dorsal marginal zone of late blastula [stage 9.5, (A)] Xenopus embryos. In early gastrula (stage 10.25), Xegr-1-expressing cells are concentrated in the area forming the dorsal blastopore lip [(B), arrow]. As gastrulation proceeds, this primary domain of Xegr-1 expression extends laterally [stage 10.5, (C)] until it defines a ring-like area around the yolk plug [stage 10.75, (D)]. In advanced gastrula [stage 12.5, (E)], expression around the small yolk plug is maintained and begins to extend into the dorsal midline area. Xegr-1 expression in the anterior head structure of tadpole (stage 32) Xenopus embryos is illustrated in (F).
Fig. 3. Comparative analysis of the spatial and temporal expression characteristics of Xegr-1 and other early mesodermal marker genes during gastrulation in *X.laevis*. (A) Total RNA preparations from staged *Xenopus* embryos were analysed for their relative content of Xegr-1, Xbra, eomes, Xvent-1 and gsc mRNAs by quantitative RT-PCR assays. Co-amplification of histone H4 served as internal control. In this experiment, Xegr-1 was amplified for 28 cycles to reveal the maternal mRNA component. (B) Adjacent sections prepared from late blastula (stage 9), early (stage 10.5) and late (stage 12.5) gastrula embryos were analysed for eomes, Xbra, chd and Xegr-1 transcript distribution by use of *in situ* hybridization. For all sections on display, the animal pole is located at the top and dorsal is on the right hand side. The lower part of the figure represents magnifications of the dorsal and ventral blastoporus from the stage 12 *Xenopus* embryo.
subfamily of zinc finger proteins that is characterized by a virtually identical cluster of three zinc finger modules in tandem repeat. egr-1 was identified first as an immediate early response gene that can be induced by a variety of stimuli including FGFRs (reviewed in Gashler and Sukhatme, 1995). The mammalian egr-1 gene product has been found to contain four SREs within 0.6 kb of its 5′-flanking sequence, a region which is sufficient to maintain full inducibility in transient transfection assays (Christy et al., 1988; Christy and Nathans, 1989; Janssens-Timmen et al., 1989). Transcriptional regulation via these SREs is thought to be mediated by a ternary complex factor-dependent mechanism that makes use of SRF and an Ets-related protein (McMahon and Monroe, 1995; Robinson et al., 1997). The Egr-1 protein is a DNA-binding transcriptional activator (Lemaire et al., 1990) that can be regulated negatively by specific co-repressor proteins referred to as NAB1 and NAB2 (Russo et al., 1995; Svaren et al., 1996). Targeted disruption of the murine egr-1 gene reveals no obvious defects during embryonic development, but was found to result in infertility (Lee et al., 1996; Topilko et al., 1998).

With the description of structure and expression of the Xenopus egr-1 homologue (Xegr-1), we here present the identification of the first C2H2-type zinc finger transcription factor that is expressed in the Spemann organizer region. A detailed comparative analysis of its temporal and spatial expression characteristics during gastrulation reveals a very close correlation with the expression pattern of Xbra. However, even though both genes can be induced by the same set of signalling molecules, i.e. activin, BMP-4 and eFGF, they are subject to control by distinct transcription factors. Induction of both Xbra and Xegr-1 gene transcription can be modulated via the MAPK signal transduction pathway; however, in contrast to induction of Xbra, induction of Xegr-1 is prevented by a dominant-negative mutant version of the Ets family transcription factor Elk-1. In addition, an activated form of SRF can stimulate Xegr-1 but not Xbra gene activity. The existence of conserved, functional SRF- and Ets-binding sites in the promoter of the Xegr-1 gene provides a further indication for a direct role of SRE–SRF–Ets ternary complexes in the regulation of Xegr-1 gene transcription during mesoderm induction in Xenopus. Finally, we demonstrate that ectopic Xbra can activate Xegr-1 transcription in animal caps (ACs); this activation occurs indirectly, most likely via Xbra-induced synthesis of an FGF-type signalling molecule.

Results

Isolation and structural characterization of Egr-1-encoding cDNA from Xenopus laevis

An RT–PCR-based cloning approach making use of degenerate oligonucleotide primers directed at the most highly conserved sequence elements in a comparison of all known Egr-type zinc finger proteins was used to isolate Egr-1 and Egr-3 (not discussed) encoding cDNAs from Xenopus laevis. A comparative analysis of the amino acid sequence predicted for Xegr-1 with the corresponding human and zebrafish sequences is shown in Figure 1. The overall degree of sequence identity of these three vertebrate proteins is high, with the most highly conserved portion being defined by the cluster of the three consecutive zinc finger modules and a directly adjacent basic region. This domain of the protein has been found to be responsible for DNA-binding activity and nuclear transfer of Egr-1 (Gashler et al., 1993; Matheny et al., 1994). Certain regions within the N-terminal halves of the proteins, which define an extensive activation domain (Gashler et al., 1993; Carman and Monroe, 1995), also reveal a high degree of conservation. A stretch of glycine and serine residues close to the N-terminus that typifies the human Egr-1 sequence is missing in the Xenopus sequence, as is the case with the zebrafish protein (Drummond et al., 1994); on the other hand, an insertion of 11 amino acids...
that maps in the N-terminal portion of the zebrafish protein is not present in the other vertebrate sequences characterized to date. The C-terminus of the Egr-1 protein that may also function in transcriptional regulation (De Franco et al., 1993; Gashler et al., 1993; Carman and Monroe, 1995) exhibits the highest degree of sequence variability. A 34 amino acid inhibitory domain that was demonstrated to interact with either one of two structurally related co-repressor proteins (Russo et al., 1995; Svaren et al., 1996) is only moderately, yet significantly conserved.

In summary, the overall high degree of structural conservation as defined by primary sequence comparison of the different vertebrate Egr-1 proteins suggests that Xegr-1 functions as a sequence-specific DNA-binding transcriptional activator, similar to its homologues from different mammalian species and from fish (reviewed in Gashler and Sukhatme, 1995).

**Spatial and temporal characteristics of Xegr-1 gene transcription during Xenopus development**

In whole-mount *in situ* hybridization analyses, Xegr-1 expression can first be detected in the dorsal marginal zone of blastula stage embryos (Figure 2). At the onset of gastrulation, Xegr-1-expressing cells are detected exclusively within the Spemann organizer region; as gastrulation proceeds, the *Xegr-1* expression domain expands and defines a ring-like domain around the yolk plug. This primary region of *Xegr-1* gene transcription is maintained in advanced gastrula stage embryos, with weak expression also extending into the dorsal midline area. During neurulation, localized *Xegr-1* mRNA was not detected; in late tailbud stages of *Xenopus* embryogenesis, *Xegr-1* expression identifies two symmetrically located spots that could be located in the anterior forebrain and which appear to be connected via a bridge of *Xegr-1*-expressing cells.

A comparative expression analysis of *Xegr-1* and several other genes, which are activated during early mesodermal patterning, was performed using RT–PCR analysis with total RNA preparations from staged embryos and *in situ* hybridization with sections prepared from late blastula (stage 9) to early (stage 10.5) and advanced (stage 12.5) gastrula embryos. RT–PCR analysis of the temporal *Xegr-1* expression profile during early embryogenesis (Figure 3A) reveals a low level maternal contribution; *Xegr-1* mRNA strongly increases with the onset of gastrulation and is found to decrease again in later stages of embryonic development. RNA preparations from adult organs and tissues exhibit comparable levels of *Xegr-1* mRNA in all samples tested (data not shown). The early embryonic expression of all other mesodermal markers analysed, such as *Xbra*, *eomes*, *Xvent-1* and *gsc*, is transient and appears to start during late blastula/early gastrula stages of development.

In whole-mount *in situ* hybridization studies on serial sections (Figure 3B), localized *Xegr-1* mRNA can first be detected at stage 9.5, when it appears to be restricted to the dorsal marginal zone, very similarly to chordin mRNA. *eomes* mRNA is found to be transcribed in the same area, but is also expressed in the ventral marginal zone. At the onset of gastrulation (stage 10.5), *Xegr-1* gene expression increases dorsally and a low level of transcription can also be detected in the ventral mesoderm. *Xbra* is expressed in a similar fashion within dorsal and ventral mesoderm. In contrast, *chd* transcripts remain restricted to the dorsal side of the embryo. For *eomes*, the dorsal and, to some extent, also the ventral expression domains are significantly wider than those of the other genes, extending well into
the animal hemisphere of the embryo. As gastrulation proceeds (stage 12.5), these differential expression characteristics of the four genes analysed are strongly maintained.

A closer inspection of dorsal and ventral involuting mesoderm of stage 12.5 embryos reveals additional, more subtle differences. On the dorsal and ventral side of the embryo, Xegr-1 is expressed in both superficial and deep cell layers. Xbra appears to be transcribed more weakly in superficial than in deeper cell layers on the dorsal side of the embryo and is excluded from the outermost cell layer on the ventral side. However, just like Xegr-1, Xbra expression is restricted to the centre of the organizer area. chd mRNA appears to be distributed uniformly between superficial and deep cell layers, but extends far beyond the centre of the organizer in the direction of the animal pole, which is the most distinguishing characteristic in comparison with the other genes analysed here.

In summary, Xegr-1 represents a novel gene expressed in the Spemann organizer territory. The spatial expression characteristics of Xegr-1 during gastrulation are paralleled most closely by those of Xbra, and they differ significantly from those of chd and eomes.

Induction of Xegr-1 by FGF and TGF-β-type peptide growth factors in animal cap explants depends on MAPK signalling

To identify signalling molecules able to induce Xegr-1 expression during mesoderm induction, we made use of the AC system. Microinjection of mRNAs encoding either eFGF, activin or BMP-4 induces Xegr-1 activity in AC explants (Figure 4A–C, compare lanes 1 and 2). An identical induction profile was observed with Xbra. In order to investigate a potential interdependence of the signalling mechanisms inducing these genes, we made use of dominant-negative variants of both the FGF and the activin receptor. Ectopic expression of dominant-negative FGF receptor (dnFR) results in a complete inhibition of Xegr-1 induction by either XeFGF, activin or BMP-4 (Figure 4A–C, compare lanes 2 and 4). The same was generally true for Xbra expression, with the exception of BMP-4 signalling which was only partially, though significantly reduced by dnFR. Xvent-1 expression, when induced by BMP-4, was found to be unaffected by dnFR (data not shown), providing an important control which reveals that dnFR did not act unspecifically or in a generally toxic fashion. Ectopic expression of dominant-negative activin receptor (dnAR) had no inhibitory effect on Xegr-1 induction by eFGF (Figure 4A, lane 5), whereas activation by activin or BMP-4 was abolished (Figure 4B and C, lanes 5). With regard to Xbra expression, once again, all dnAR effects mirror those observed with Xegr-1, with the exception of the BMP-4 response, which was only partially reduced.

We conclude that Xegr-1 induction by TGF-β- and FGF-type peptide growth factors strictly depends on a functional FGF signalling pathway. This is similarly true for Xbra, with the exception of BMP-4-mediated activation, which we find to be only partially reduced by dnFR.

The strict requirement for FGF signalling for Xegr-1 gene expression suggests an involvement of MAPK signalling in this process. We therefore co-injected mRNA encoding the MAPK phosphatase-1 (MKP-1), which specifically dephosphorylates and inactivates MAPKs (Gotoh et al., 1995; La Bonne et al., 1995). Indeed, MKP-1 overexpression exerted the same overall effects on expression of either Xegr-1 or Xbra as dnFR overexpression (Figure 4A–C, lanes 6). This clearly implicates the MAPK signalling activity as being essential for Xegr-1 regulation. Further support for this notion comes from experiments with a constitutively active variant of Ras (caRas), which has been shown to be a highly efficient upstream activator of MAPKs (La Bonne et al., 1995). The caRas molecule was found to induce both Xegr-1 and Xbra transcription in ACs (Figure 5A, lane 3), an effect that could be fully inhibited by co-expression of a dominant-negative version of Raf (dnRaf) which operates downstream of Ras (Figure 5A, lane 5).

Taken together, the above results demonstrate that both Xegr-1 and Xbra are modulated similarly by FGF-induced MAPK signalling in the AC assay system. This shared feature of transcriptional regulation may be the underlying cause of the overlapping expression patterns of Xegr-1 and Xbra as observed in the comparative analysis of the spatial expression characteristics described above.

Inhibition of TCF/Ets transcription factor activity and activation of SRF affect Xegr-1 expression in animal cap assays

Ets protein family transcription factors can function as direct nuclear targets of FGF-induced MAPK activities (for review see Wasylyk and Nordheim, 1997). To investigate a potential involvement of Ets proteins in mediating the induction of Xegr-1 and other mesodermal marker genes by FGF, activin or BMP-4, we co-expressed a dominant-negative version of the Ets protein Elk-1 (dnElk-1). dnElk-1 inhibits the activity of SRF-interacting Ets proteins since it maintains DNA binding specificity while lacking the C-terminal transcriptional activation domain, which normally is activated upon phosphorylation by MAPKs (Janknecht et al., 1993; Marais et al., 1993). dnElk-1 specifically inhibits Xegr-1 expression, irrespective of the inducer utilized (Figure 4A–C, compare lanes 2 and 7). In contrast, induction of Xbra was not reduced. In order to attribute this effect of dnElk-1 more directly to defective phosphorylation of Elk-1, we employed single and triple point mutant versions of Elk-1 [Elk-1(A) and Elk-1(5AAA)], which have crucial MAPK substrate serines replaced by alanines (Janknecht et al., 1993). AC assays reveal that the inhibitory effect of dnElk-1 on FGF-induced Xegr-1 expression can be reproduced by Elk-1(A) or Elk-1(5AAA) (Figure 5B, lanes 6–8), while Elk-1 wild-type protein did not exert any influence (Figure 5B, lane 5). The expression of Xbra remained largely unaffected by any of these Elk-1 variants.

These results suggest that activation of an endogenous Ets protein is involved in the signal-induced regulation of Xegr-1 expression. In contrast, the involvement of such Ets factors in Xbra expression is unlikely, unless they involve Ets proteins with DNA binding specificities distinct from that of Elk-1. The differential sensitivity of Xegr-1 and Xbra towards dominant-negative Elk-1 proteins argues for a mechanistically different mode of transcriptional regulation at a level downstream of MAPKs.

Elk-1 and other TCF-type Ets proteins relay MAPK
Fig. 6. The Xegr-1 gene promoter contains multiple, conserved SRF-binding sites (CArG-boxes) and Ets-binding sites (EBSs). (A) DNA sequence of a genomic clone partially representing the Xegr-1 gene. The position of conserved CArG-boxes (boxed) and EBSs (underlined) is indicated. (B) The position of SRF- and Ets-binding sites in different vertebrate egr-1 genes. Analysis of 5′/H11032-flanking sequences was performed with the corresponding Xenopus (this study), mouse (Janssen-Timmen et al., 1989), rat (Changelian et al., 1989), human (Suggs et al., 1990; Sakamoto et al., 1991) and zebrafish (Drummond et al., 1994) DNA fragments. (C) Ternary complex formation with conserved sequence elements derived from the Xegr-1 promoter region. A synthetic oligonucleotide probe corresponding to position –425 to –389 of the Xegr-1 5′-flanking region was incubated with SRF or Elk-1 protein, or with a combination of both proteins. The positions of complexes forming with SRF (C1) and of the ternary complex forming with both proteins (C2) are indicated. mSBS and mEBS oligonucleotide probes contain single site mutations in the SRF- and Ets-binding sites, respectively. An oligonucleotide probe derived from the c-fos gene promoter (position –276 to –299) was used as a positive control for SRF binding and ternary complex formation. The arrowhead indicates an as yet uncharacterized complex that forms using high amounts of Elk-1 and probably consists of two Elk-1 molecules. The asterisk indicates unbound oligonucleotides.

signals to target promoters via interaction with and recruitment by the transcription factor SRF (reviewed in Treisman, 1996; Wasylyk and Nordheim, 1997). We therefore investigated the potential involvement of SRF in transcriptional induction of Xegr-1. For this purpose, we made use of a constitutively active derivative of SRF (SRF–VP16) which contains a fused VP16-derived transactivation domain rendering SRF independent of Ets protein activity. SRF–VP16 is indeed able to stimulate Xegr-1 expression in ACs in the absence of any mesoderm inducers, while wild-type SRF (XSRF) or the VP16 domain fused to the DNA-binding domain of the unrelated MyoD protein (MyoD–VP16) are unable to do so (Figure 5C, lanes 4 and 6; data not shown). In great contrast, SRF–VP16 does not induce Xbra expression. A further difference between Xegr-1 and Xbra is observed in that Xegr-1 induction by eFGF is enhanced upon co-injection of SRF–VP16, whereas Xbra induction by eFGF is inhibited in the presence of SRF–VP16 (Figure 5C, lanes 3 and 5). We attribute this latter effect to non-specific squelching (Ptashne, 1988), because MyoD–VP16 exhibits a similar negative effect on Xbra induction by eFGF (data not shown).

Taken together, these observations lead us to propose
that the regulation of Xegr-1 transcription involves Ets-type proteins and SRF. Such a situation could reflect a direct cooperation of these transcription factors on the Xegr-1 promoter.

**The Xegr-1 gene promoter contains functional SRF–Ets-binding sites**

To analyse further if the observed regulatory effects on Xegr-1 transcription exerted by the activity of SRF- and Ets-type proteins might reflect direct interactions with the Xegr-1 gene, we have isolated and characterized a genomic fragment from *X.laevis* that contains part of the Egr-1-coding region, the promoter and ~3 kb of 5′-flanking sequence (Figure 6A). Like its mammalian homologues, the Xegr-1 gene contains an intron in a conserved position. Sequence analysis of the direct 5′-flanking region reveals the presence of three potential SRF-binding sequences, located in conserved positions with regard to the different vertebrate egr-1 genes described previously (Figure 6B). Two SRF-binding sites are located close to the start site of transcription, and a conserved Ets-binding site is positioned between these two proximal SRF-binding sites. A third conserved SRF-binding site is located ~300 nucleotides further upstream. This element is flanked by multiple Ets-binding sites, one located four nucleotides downstream, a second one three nucleotides upstream. Curiously, even though the pattern defined by the relative positions of these multiple SRF- and Ets-binding sites is reflected in all five vertebrate egr-1 genes compared, none of the five different SRF-binding sites is absolutely conserved. In contrast, four Ets-binding sites are present in conserved positions of all five genes (Figure 6B).

A region of the Xegr-1 distal promoter, containing one SRF- and three Ets-binding sites (nucleotide position −389 to −425), was tested for its ability to interact with the SRF and Elk-1 proteins from mammalian sources (Figure 6C). SRF alone was capable of forming a specific binary complex with a synthetic oligonucleotide covering the critical region (C1). Single-site mutation within the SRF consensus-binding site abrogated complex formation. Conversely, the Elk-1 protein by itself was not able to bind the SRE sequences alone; in combination with SRF, however, formation of a ternary complex could be detected (C2). Mutations within the Ets-binding site maintain the ability of the oligonucleotide to interact with SRF but prevent ternary complex formation.

Thus, a sequence derived from the 5′-flanking region of the Xegr-1 gene is functional in forming a specific DNA–protein complex with the transcription factors SRF and Elk-1. Together with the results obtained with dnElk-1 and activated SRF in ACs (as described in the previous section), these observations strongly suggest a direct regulatory function for SRF and Ets-type proteins in the FGF signal transduction pathway rather than by direct action of the Xbra protein on the Xegr-1 promoter. To this end, Xbra was expressed along with agents that were designed to interfere with either FGF or activin signalling. In the presence of either dnFR or MKP-1, Xbra was no longer capable of activating Xegr-1 transcription; dnAR has no effect on the same process (Figure 7, lanes 5–7). These results suggest an indirect mechanism as being responsible for Xbra-mediated Xegr-1 activation, a process initiated via Xbra-induced FGF production.

**Discussion**

The description of the expression characteristics of the egr-1 homologue in *X.laevis* identifies the first C2H2-type zinc finger protein found to be expressed in the Spemann organizer region. As demonstrated for other organizer-expressed genes, Xegr-1 expression can be induced by dorsalizing and ventralizing peptide growth factors. Xegr-1 gene transcription responds to the FGF-induced MAPK/SRF–Ets signal transduction pathway. Activation of Xegr-1 expression by activin and BMP-4 is achieved by an indirect mechanism that appears to operate via Xbra-induced synthesis of FGF.

** Territories of organizer-specific gene expression**

Regional gene expression characteristics between the late blastula and early gastrula stages of *Xenopus* embryo-ogenesis differ in detail for the different organizer genes that have been analysed. Even though their primary
expression domains within the dorsal marginal zone vastly overlap, and even though they respond to an overlapping set of mesoderm-inducing signalling molecules, they appear to define distinct organizer territories in the animal–vegetal and superficial–deep dimensions (Vodicka and Gerhart, 1995). This situation is indicative of a more complex set of molecular mechanisms being responsible for generating these distinct patterns of transcriptional responses.

Indeed, the three different AREs that have been characterized to date share no obvious structural homology (Huang et al., 1995; Watabe et al., 1995; Kaufmann et al., 1996). Furthermore, none of the corresponding promoters has been described to carry functional SREs, which we propose to mediate the peptide growth factor responsiveness of the organizer-expressed Xegr-1 gene. It will be interesting to compare the minimal sets of regulatory elements that can give rise to the precisely regulated expression characteristics of organizer-specific genes; it seems probable that these will include positive and negative regulatory elements as was first shown to be the case for the XPD-1 gene in the frog (Kaufmann et al., 1996).

The question has been raised as to whether these different organizer expression domains reflect different functional domains (Vodicka and Gerhart, 1995). Our attempts to characterize the biological function of Xegr-1 further by injection of mRNAs encoding either wild-type or putative dominant-negative versions of Xegr-1 into Xenopus embryos have not resulted in the generation of informative effects; similarly, inhibition of Xegr-1 expression by dnElk-1 did not inhibit activin- or eFGF-induced elongation of AC explants (data not shown). These observations are in line with the finding that Egr-1-deficient mice did not exhibit obvious developmental defects during embryogenesis (Lee et al., 1996; Topilko et al., 1998).

**Mesoderm induction and MAP kinase activity**

A number of investigations have identified elements of the MAPK signal transduction pathway to be critically involved in FGF-mediated mesoderm induction (Whitman and Melton, 1992; MacNicol et al., 1993; Hartley et al., 1994; Gotoh et al., 1995; Tang et al., 1995) and, more specifically, in the activation of Xbra gene transcription (La Bonne et al., 1995; Northrop et al., 1995; Umbhauer et al., 1995). Moreover, it has been demonstrated that in addition to this function in early mesoderm, FGF serves a second function during later stages of gastrulation, where it maintains expression of Xbra. This appears to be achieved via an autoregulatory loop in which Xbra directly induces expression of eFGF (Isaacs et al., 1994; Schulte-Merker and Smith, 1995; Tada et al., 1997).

Findings from the AC experiments presented here strongly suggest that in addition to Xbra, the Xegr-1 promoter is a further target for FGF signalling during mesoderm induction. Furthermore, our results also indicate that Xbra induces Xegr-1 indirectly, via activation of FGF production. Such a scenario would imply that the autoregulatory Xbra–eFGF loop not only serves to maintain expression of genes which are already in the process of being actively transcribed (i.e. Xbra), but the same loop would also serve to induce novel gene activity in the mesoderm (i.e. Xegr-1). When ACs were incubated directly with activin protein, induction of Xbra preceded that of Xegr-1 by 2 h (data not shown). Taken together, these arguments led us to propose a model (Figure 8) of how Xegr-1 expression could be regulated during mesoderm induction in Xenopus embryos. We currently are investigating whether Xegr-1 expression requires *de novo* protein synthesis in order to be able to respond to FGF, activin or BMP-4 treatment. If our model is correct, we would expect Xegr-1 expression to be cycloheximide-sensitive with respect to activin and/or BMP-4, but not to FGF induction.

The molecular cascades of Xegr-1 and Xbra transcriptional regulation appear to share components of the FGF signal transduction pathway reaching from the FGF receptor to MAPK. However, downstream of MAPK, different transcription factors appear to be responsible for transmitting the activating stimulus via gene-specific promoter elements. Our findings suggest that an Ets-type protein together with SRF is directly responsible for FGF-induced Xegr-1 induction. Conversely, FGF-induced Xbra induction seems to be regulated by transcription factors different from SRF-interacting Ets proteins. Our results are in agreement with the recent characterization of the FGF-responsive promoter fragment in the Xbra2 gene, which does not appear to contain any SRE-related sequence elements (Latinkic et al., 1997).

**Materials and methods**

**cDNA isolation**

Based on Egr-1, -2 and -3 sequences from mouse, rat and human, degenerate primers for conserved sequence elements were designed. Nested PCR on X.laevis genomic DNA was performed using outer primers that correspond to amino acid sequences found in all Egr proteins (PMIPDY EGR2-2F, 5'-CCTAGATCTCCGAYTAC; GHKPFQC HC-2R, 5'-TCRCAYTGRAGYTTTGC; and inner primers specific for the Egr-1 subfamily located in the non-zinc finger region (FPOQQGD EGR1-3F, 5'-TYCCACCCARCARCARGGIGA; KTPVHE EGR1-4R, 5'-CKYCTCCTGGIGGGGCTTT). PCR products were cloned into pBluescriptII KS (Stratagene) and sequenced. An oligo(dT) λ, ZAP Express stage 30/32 tail cDNA library was screened using Xegr-1-specific primers derived from the corresponding genomic fragment. A 2807 bp cDNA clone was isolated and sequenced in both directions using Sequenase (US Biochemical) or Taq dye terminator cycle sequencing (Applied Biosystems).
**Cloning of Xegr-1 promoter fragment**

An *X.laevis* genomic A Fix II library was screened by PCR with primers located in the 5′ region of the *Xegr-1* cDNA. Hybridization with a digoxigenin-labeled DNA probe produced by PCR from the *Xegr-1* cDNA clone identified a positive genomic clone. A 4116 bp HindIII fragment was subcloned into pGEM-3Zf(+) (Promega) and sequenced in both directions.

**In situ hybridization procedures**

Whole-mount in situ hybridization was performed in principle as described by Harland (1991) using digoxigenin-11-UTP-labeled anti-sense RNA probes. The *Xegr-1* antisense RNA probe was prepared by linearizing a full-length pHKCMV-Xegr1 with SalI (2.8 kb probe) and BamHI (1.6 kb probe) and transcription with T7 polymerase. DIGoxigenin-labeled hybrids were visualized by alkaline phosphatase-conjugated anti-digoxigenin Fab fragments and BM Purple substrate or NBT/BCIP.

**Handling and manipulation of X.laevis embryos**

Eggs were collected from *X.laevis* females (Xenopus 1, Ann Arbor, MI) which had been injected with 400–600 U of human chorionic gonadotrophin (Sigma) 10–12 h prior to egg collection. Eggs were fertilized with mixed testes, incubated in 0.1x modified Barth saline (XN) and staged according to Nieuwkoop and Faber (1967).

For AC assays, embryos were dejellied in 2% cysteine hydrochloride (pH 7.6) and injected with synthetic mRNA into each blastomere at the four-cell stage. mRNA was prepared from linearized plasmids using SP6 polymerase (mMessage mMACHINE, Ambion). A total of 10 nl of mRNA solution was injected into the animal pole at 30–35 s.p.i. and 40–60 ms (for amounts of injected mRNA, see below). ACs were prepared from stage 10 embryos. Five ACs were pooled and lysed immediately in 100 μl of Tris-buffered saline (AGS, Heidelberg). Each embryo was lysed in 100 μl of Tristar.

**Quantification of gene expression by RT–PCR**

The procedure of Rupp and Weintraub (1991) was modified as follows. Total RNA was isolated from Tristar lysates of ACs or embryos, according to the manufacturer’s instructions (Ags). RNA of one AC was dissolved in 2 μl of RNase-free water, whereas RNA of one embryo was dissolved in 25 μl of RNase-free water. The reverse transcription reaction was performed using the ‘first strand buffer’ from Gibco-BRL.

PCRs were done with 1 U of Taq polymerase (Eugonecent) and 1 μCi of [α-32P]dATP (3000 Ci/mmol; Hartmann, Braunschweig). PCR profile: denaturation for 30 s at 94°C, primer annealing for 30 s at 58°C, and elongation for 60 s at 72°C. One-fifth of the PCR sample was electrophoresed on 8% polyacrylamide gels, and the PCR fragments were visualized by autoradiography (exposure 7 h, Fuji phosphorimage). The amount of PCR product was quantified by radioactive nucleotide incorporation, followed by normalization over the H4 RT–PCR signals.

**Characteristics of Xegr-1 expression in *X.laevis***

PCR primers

H4: 5′-CCGATGATACCAGGTTATCT-3′ (F); 5′-ATCATGGC- GGAATCTCTCTCT-3′ (R); 188 bp fragment, 19 cycles (Niehrs et al., 1994). Xba: 5′-GACAGTGTGATGGT- TCTTTGAC-3′ (F); 5′-ACAAAGTCGACAGAAGGC-3′ (R). Positions 119–415; 297 bp fragment, 25 cycles (Smith et al., 1991).

**Electrophoretic mobility shift assay**

End-labelled, double-stranded oligonucleotides (30 000 c.p.m. per reaction) were used in each DNA binding reaction. Binding conditions: 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA; 0.05% (w/v) milk powder, 5% (v/v) glycerol, 10 mM dithiothreitol (DTT), 2 μg of poly(dI-dC)–poly(dI-dC) and radioactive oligonucleotide (30 min at room temperature; 10 μl reaction volume). This was supplemented with 2 μl of purified human SRF protein (SRF–6His; Janknecht et al., 1991) and/or 4 μl of purified Elk-1 protein (N6His-Elk-1; Janknecht and Nordheim, 1991). For loading on a 4% polyacrylamide/0.5× TBE gel, samples were mixed with 2.5 μl of 10% Ficoll 400, 15% glycerol, 40 mM EDTA. Electrophoresis was performed at 700 V for 1 h at 15°C.

**Template plasmids for mRNA synthesis by in vitro transcription**

cDNAs, cloned into either pSP64T (Krieg and Melton, 1984; Melton et al., 1984) or pc2+/+ (Rupp et al., 1994), were used as templates for in vitro transcription from linearized plasmids. Restriction enzymes used for linearization (lin.) are indicated only for plasmid constructs generated here. Standard amounts of injected mRNA per embryo were as indicated in parentheses, unless stated otherwise in the figure legend. pSP64T/XefGF (Isaacs et al., 1992) (0.5 ng); pSP64T/activin (Rodolpho et al., 1993) (1 ng); pSP64T/dnARaf (known as p6fT-XARl-DNMI/Stop) (Hemmati-Brivanlou and Melton, 1992) (1 ng); pSP64T/Xsrf (Mohun et al., 1991) (0.5 ng); pSP64T/Xbra (Cunliffe and Smith, 1992) (0.5 ng); pSP64T/BMP-4 (Koster et al., 1991) (1 ng); pSP64T/dnARaf (known as p6fT-XAF1) (Amaya et al., 1991) (1 ng); pSP64T/dnARaf (known as p6fT-XARl-DNMI/Stop) (Hemmati-Brivanlou and Melton, 1992) (1 ng); pSP64T/Xsrf (Mohun et al., 1991) (0.5 ng).


