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

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**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*.
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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 *gooseoid* (*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

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; 3/4, 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).

of the Ets protein family (Elk-1, Sap1a and Net; reviewed in Treisman, 1996; Wasylyk and Nordheim, 1997). The TCF-type Ets proteins interact with serum response factor (SRF) to form ternary complexes at transcriptional control elements termed serum response elements (SREs) (Shore and Sharrocks, 1994; Wasylyk and Nordheim, 1997). SREs regulate the immediate early transcriptional activation response of mammalian genes like *c-fos*, *α-actin* and *egr-1* (Herschman, 1991). SRE-mediated gene activation can be downmodulated efficiently by dominant-negative versions of TCF molecules that have lost or mutated their MAPK phosphorylation sites (Janknecht *et al.*, 1993; Marais *et al.*, 1993).

Transcriptional regulation by SRF has been implicated in mesoderm-specific gene expression; in the developing *Xenopus* embryo, muscle-specific expression of the cardiac actin gene is under the control of SRF (Mohun *et al.*, 1989, 1991). Like the mammalian *Egr-1* gene, the cardiac actin gene promoter carries multiple SRF-binding sites (CARG-boxes), the most proximal of which is required for mesoderm-specific gene activation (Mohun *et al.*, 1989).

Egr-1 is member of a small, evolutionarily conserved

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.

Fig. 4. *Xegr-1* gene transcription can be induced by dorsalizing and ventralizing peptide growth factors. AC assays measuring the effects of mesoderm-inducing signalling molecules (XeFGF, activin, BMP-4) on the expression of *Xegr-1* and *Xbra* were performed in the presence or absence of dominant-negative FGF receptor (dnFR), dominant-negative activin receptor (dnAR), MAPK phosphatase 1 (MKP-1) and dominant-negative Elk-1 (dnElk-1), as indicated. (A) RT-PCR analysis of ACs derived from embryos injected with XeFGF mRNA (lanes 2–7) in the presence of co-injected mRNAs dnFR (lane 4), dnAR (lane 5), MKP-1 (lane 6) or dnElk-1 (lane 7). Control samples were taken from ACs of uninjected embryos (lane 1) or from XeFGF mRNA-injected embryos without co-injections (lanes 2 and 3). Genomic DNA contamination was assayed for in a PCR control amplification which was missing a prior reverse transcription step (RT–, lane 3). The endogenous embryonic expression at the time of AC removal is assayed in lane 8. A template-free RT-PCR control is given in lane 9. Specific RT-PCR primer sets were used to measure the expression of *Xegr-1* and *Xbra*. Each reaction included primers for co-amplification of histone H4 mRNA. (B) As in (A), except that AC stimulation was induced with injected activin mRNA. The mRNAs for dnFR, dnAR and MKP-1 were injected at 2 ng per embryo. (C) As in (A), except that AC stimulation was induced with injected BMP-4 mRNA. The dnAR mRNA concentration used was 2 ng.

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 FGFs (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; Janssen-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

Fig. 5. *Xegr-1* gene transcription is regulated via the MAP kinase/SRF-Ets-mediated signal transduction pathway. (A) Ras signalling can induce *Xegr-1* expression in ACs. *Xegr-1* and *Xbra* are induced in ACs derived from embryos injected with mRNA encoding a constitutively active variant of Ras (caRas, lane 3), but not if mRNA encoding a dominant-negative version of the downstream kinase Raf (dnRaf) is co-injected (lane 5). Controls were as described in Figure 4. (B) Dominant-negative versions of the TCF/Ets protein Elk-1 can inhibit XeFGF-induced *Xegr-1* but not *Xbra* expression. ACs from embryos injected with XeFGF mRNA express *Xegr-1* and *Xbra* (lane 3), which is unaffected by co-injection of Elk-1 mRNA (lane 5). In contrast, *Xegr-1* expression is inhibited upon co-injection of mRNA encoding a dominant-negative derivative of Elk-1 (dnElk-1, lane 6) or Elk-1 mutated in one or three crucial phosphorylation sites [Elk-1(A) and Elk-1(AAA), lanes 7 and 8, respectively]. All of these co-injected mRNAs left expression of *Xbra* largely unaffected (lanes 5–8). Controls were as described in Figure 4. (C) Activated SRF (SRF-VP16) specifically induces *Xegr-1* expression in the absence of mesoderm-inducing peptide growth factors. ACs derived from embryos injected with mRNA encoding XeFGF exhibit expression of both *Xegr-1* and *Xbra* (lane 3); SRF-VP16 is able specifically to induce *Xegr-1* but not *Xbra* (lane 4). This effect on *Xegr-1* gene transcription can be enhanced by co-expression of XeFGF; conversely, SRF-VP16 inhibits XeFGF-mediated induction of *Xbra* (lane 5). Wild-type SRF (XSRF) is unable to cause any stimulation by itself (lane 6).

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 activation (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* expression is restricted to the deep cell layers, i.e. the involuted dorsal mesoderm. *eomes* 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 Wasyluk 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(AAA)], 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(AAA) (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 EBSSs (underlined) is indicated. **(B)** The position of SRF- and Ets-binding sites in different vertebrate *egr-1* genes. Analysis of 5'-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 consist of two Elk-1 molecules. The asterisk indicates unbound oligonucleotides.

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).

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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 eFGF-mediated induction of *Xegr-1* transcription.

Ectopic expression of *Xbra* can activate *Xegr-1* via a mechanism that involves FGF signalling

The observed close correlation of the *Xegr-1* and *Xbra* expression characteristics during gastrulation (see above), as well as the fact that both genes encode transcription factors, led us to speculate that one gene might participate directly or indirectly in transcriptional regulation of the other. In order to test for this possibility, *Xbra* and *Xegr-1*

Fig. 7. *Xbra* can induce *Xegr-1* gene activity via the FGF signal transduction pathway. ACs derived from embryos injected with mRNA encoding *Xbra* exhibit *Xegr-1* gene expression (lane 3). Co-injection of negative effectors of MAPK signalling pathways, such as dominant-negative FGF receptor (dnFR, lane 5) or MAPK phosphatase-1 (MKP-1, lane 7), inhibits *Xbra*-mediated *Xegr-1* induction. Dominant-negative activin receptor (dnAR, lane 6) does not exert a negative influence. Controls were as described in Figure 5. All mRNAs were injected at 2 ng per embryo.

were ectopically expressed in ACs. It turns out that *Xbra* can activate *Xegr-1* transcription (Figure 7, lane 3), whereas *Xegr-1* cannot activate *Xbra* gene activity (data not shown). Since it has been demonstrated previously that *Xbra* can activate FGF expression (Isaacs *et al.*, 1994; Schulte-Merker and Smith, 1995), and since *Xegr-1* is regulated via the eFGF signal transduction pathway (see above), we wished to investigate whether *Xbra*-induced *Xegr-1* expression was achieved indirectly via 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* embryogenesis 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 *XFD-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

Fig. 8. A model outlining the transcriptional regulation of *Xbra* and *Xegr-1* by peptide growth factor-induced signal transduction pathways; see text for further details.

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 EGR1-2F, 5'-CCATGATCICGAYTAC; GHKPFQC HC-2R, 5'-TCRCAYTGRAAIGGYTTITGIC) and inner primers specific for the *Egr-1* subfamily located in the non-zinc finger region (FPQQQGD EGR1-3F, 5'-TTYCCICARCARGGIGA; KTPVHE EGR1-4R, 5'-CKYTCRTGIGGIGGIGTYTT). 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 λ Fix II library was screened by PCR with primers located in the 5' region of the *Xegr-1* cDNA. Hybridization with a digoxigenin-labelled DNA probe produced by PCR from the *Xegr-1* cDNA clone identified a positive genomic clone. A 4116 bp *Hind*III 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-labelled antisense RNA probes. The *Xegr-1* antisense RNA probe was prepared by linearizing a full-length pBKCMV-*Xegr1* with *Sal*I (2.8 kb probe) or *Bam*HI (1.6 kb probe) and transcription with T7 polymerase. Digoxigenin-labelled hybrids were visualized by alkaline phosphatase-conjugated anti-digoxigenin Fab fragments and BM Purple substrate or NBT/BCIP. *In situ* hybridization on sections was performed essentially as described (Lemaire and Gurdon, 1994). Sections (12.5 μ m) were prepared from paraffin-embedded embryos with a Jung RM2065 microtome (Leica) and mounted onto TESPA-coated slides. For synthesis of *Xbra* antisense RNA, *Bgl*III-linearized pSP73-*Xbra* (Cunliffe and Smith, 1992) was transcribed with T7 polymerase. An *eomes* *in situ* probe was derived by linearizing pNTM2/Bsc (Ryan *et al.*, 1996) with *Eco*RI and transcription with T3 polymerase. The chordin antisense RNA probe was prepared by linearizing pBS(SK)59-chd (Sasai *et al.*, 1994) with *Eco*RI and transcription with T7 polymerase. For genes showing only a weak signal, the sensitivity was increased using high molecular weight polyvinyl alcohol, resulting in an enhanced alkaline phosphatase reaction (De Block and Debrouwer, 1993). Imaging of whole-mounts and mounted sections was done with a Zeiss Axiophot with Nomarski optics and a CCD camera (Sony).

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 minced testis, incubated in 0.1 $\times$ modified Barth saline (MBS) and staged according to Nieuwkoop and Faber (1967).

For AC assays, embryos were dejected 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 top at 30–35 p.s.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 μ l of Tristar solution (AGS, Heidelberg). Each embryo was lysed in 100 μ l 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 μ l of RNase-free water, whereas RNA of one embryo was dissolved in 25 μ l 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 (Eurogentec) and 1 μ Ci of [α - 32 P]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 phosphorimager). The amount of PCR product was quantified by radioactive nucleotide incorporation, followed by normalization over the H4 RT-PCR signals.

For calibration of the RT-PCR assay, the linear amplification range for each primer pair was determined using a fixed amount of NF10 cDNA. The standard cDNA input was used at sub-saturating levels, as evidenced by the fact that 2-fold amounts of cDNA showed unaltered kinetics of amplification. Finally, we ensured that no primer competition occurred in our multiplex PCR conditions. The standard RT-PCR-protocol was: 8% embryo RNA equivalent or 100% AC RNA equivalent was used per reverse transcription reaction, of which 20% was used in each subsequent PCR. After initiation of these PCRs amplifying mesodermal marker gene signals, H4-specific primers were added 4–9 cycles later, depending on the first primer pair used. All AC experiments were performed at least two to three times and found to be highly reproducible.

PCR primers

H4: 5'-CGGGATAACATTTCAGGGTATCACT-3' (F); 5'-ATCCATGGC-GGTAAGTGTCTTCCT-3' (R); 188 bp fragment, 19 cycles (Niehrs *et al.*, 1994). *Xbra*: 5'-GATctagaTCTCTGGAGTAATGAGTG-3' (F); 5'-ACAAAGTCCAGCAGAACCGTA-3' (R). Positions 119–415; 297 bp fragment, 28 cycles (Smith *et al.*, 1991). Lower case letters indicate an introduced restriction site (Rupp and Weintraub, 1991). *Xegr-1*: 5'-GAGATGTTAGCCTTGTATCTGC-3' (F); 5'-GTACTGTTGATAGT-CTTGAGGTCC-3' (R). Positions 902–1043; 142 bp fragment, 25 cycles (this study). *Xvent-1*: 5'-GCATCTCCTTGGCATAATTTGG-3' (F); 5'-TTCCCTTCAGCATGGTTCAAC-3' (R); 147 bp fragment, 23 cycles (Gawantka *et al.*, 1995). *Eomes*: 5'-GTGAAGACATTGGCACTGAG-3' (F); 5'-GGCATATACAATGGTCCATCC-3' (R). Positions 2147–2281; 141 bp fragment, 23 cycles (Ryan *et al.*, 1996). *Gsc*: 5'-AGGCACAGGACCATTCTTACCG-3' (F); 5'-CACTTTTAACCTCTT-CGTCCGC-3' (R); 264 bp fragment, 28 cycles (Blumberg *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 μ g of poly(dI-dC)-poly(dI-dC) and radioactive oligonucleotide (30 min at room temperature; 10 μ l reaction volume). This was supplemented with 2 μ l of purified human SRF protein (SRF-6His; Janknecht *et al.*, 1991) and/or 4 μ l of purified human Elk-1 protein (N6His-Elk-1; Janknecht and Nordheim, 1991). For loading on a 4% polyacrylamide/0.5 $\times$ TBE gel, samples were mixed with 2.5 μ l of 10% Ficoll 400, 15% glycerol, 40 mM EDTA. Electrophoresis was performed at room temperature in 0.5 $\times$ TBE.

The oligonucleotide band shift probes shown below contained potential SRF- and Elk-1-binding sites (bold) or mutations thereof (underlined). Human *c-fos* SRE (nucleotide positions –326 to –300 of the human *c-fos* promoter; Van Straaten *et al.*, 1983), nucleotides in italics represent introduced restriction sites: sense strand, 5'-GATC CACACAGGATGT-CCATATTAGGACATCCTGTCG-3'; antisense strand, 5'-GATCCGAC-AGGATGTCCTAATATGGACATCCTGTGTG-3'. *Xegr-1* SRE (positions –425 to –389 of the *Xegr-1* promoter): sense strand, 5'-ATCCGGAAAGCCCTTAAAGGAGCAGGAAGGAACCC-3'; antisense strand, 5'-GGGTTCTTCCTTCTGCTTTTAAAGGCTTTC-GGAT-3'. *Xegr-1* SRE/mEBS (*Xegr-1* SRE with mutated Ets-binding sites, EBS): sense strand, 5'-ATCCCTTAAAGCCCTTAAAGGAGCA-TTAATTAACCC-3'; antisense strand, 5'-GGGTTAATTAATGCTCC-TTTTAAGGCTTTAAGGAT-3'. *Xegr-1* SRE/mSBS (*Xegr-1* SRE with mutated CarG-box): sense strand, 5'-ATCCGGAAAGCACTTCAAA-TGAGCAGGAAGGAACCC-3'; antisense strand, 5'-GGGTTCTTC-TCTGCTCATTGAAGTGTCTTCCGGAT-3'.

Template plasmids for mRNA synthesis by *in vitro* transcription

cDNAs, cloned into either pSP64T (Krieg and Melton, 1984; Melton *et al.*, 1984) or pCS2+ (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 legends. pSP64T/XeFGF (Isaacs *et al.*, 1992) (0.5 pg); pSP64T/activin (Rodolpho *et al.*, 1993) (1 pg); pSP64T/BMP-4 (Köster *et al.*, 1991) (2 ng); pSP64T/dnFR (known as XFD) (Amaya *et al.*, 1991) (1 ng); pSP64T/dnAR (known as p64T-XAR1-DNMI/Stop) (Hemmati-Brivanlou and Melton, 1992) (1 ng); pSP64T/XSRF (Mohun *et al.*, 1991) (0.5 ng); pSP64T/Xbra (Cunliffe and Smith, 1992) (2 ng); pSP64T/ Δ C'RAF1 (dnRAF) (Umbhauer *et al.*, 1995) (5 ng); pSP64T/v-Ras (caRas) (Umbhauer *et al.*, 1995) (12 pg); pCS2+/SRF-VP16 [1.8 kbp 5' *Nco*I, 3' *Xba*I SRF-VP16 fragment (Dalton and Treisman, 1992) with a blunted *Nco*I site was inserted into the 5' *Stu*I site and 3' *Xba*I site of pCS2+] (0.5 ng) (lin. Asp718); pCS2+/MyoD-VP16 (Rupp *et al.*, 1994) (0.5 ng); pCS2+/MKP-1 [complete murine 1.9 kbp cDNA of MKP-1 (3CH134; Charles *et al.*, 1992) cloned into the *Eco*RI site of pCS2+] (2 ng) (lin. Asp718); pCS2+/Elk-1 [complete open reading frame from nucleotides 317 (ATG codon) to 1725 (*Bam*HI site) of the human *elk-1* cDNA (Rao *et al.*, 1989)] (0.3 ng) (lin. Asp718); pCS2+/dnElk-1 (coding region for amino acids 1–309, nucleotides 317–1238 of human *elk-1* cDNA; the 3' end of cDNA has been constructed by introducing a stop codon 3' of amino acid 309) (lin. *Nsi*I), (0.3 ng); pCS2+/Elk-1, S383A [the 5' *Bgl*III (nucleotide 1233 of *elk-1* cDNA) and 3' *Xba*I fragment of pCS2+/*elk-1* have been replaced by the 5' *Bgl*III–3' *Xba*I fragment of pEV3S/*elk-1*,

S383A (Janknecht and Nordheim, 1992; Janknecht *et al.*, 1993) (lin. Asp718) [0.3 ng; injected mRNA termed Elk-1(A)]; pCS2+/Elk-1 S324/383/389A [the 5' *Bgl*III (nucleotide 1233 of *elk-1* cDNA) and 3' *Xba*I fragment of pCS2+/elk-1 have been replaced by the 5' *Bgl*III, 3' *Xba*I fragment of pEV3S/elk-1 S324/383/389A (Janknecht *et al.*, 1993)] (lin. Asp718) [0.3 ng; injected mRNA termed Elk-1(AAA)].

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