Integration of multiple signal transducing pathways on Fgf response elements of the *Xenopus caudal* homologue *Xcad3*

Tomomi Haremaki[1,*], Yasuko Tanaka[1,2], Ikuko Hongo[1], Masahiro Yuge[3] and Harumasa Okamoto[1,†]

[1]Neuroscience Research Institute, National Institute of Advanced Industrial Science and Technology (AIST) Central 6, 1-1-1 Higashi, Tsukuba 305-8566, Japan
[2]Department of Anatomy, Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8577, Japan
[3]Faculty of Human Environmental Science, Fukuoka Women's University, Kasumigaoka Higashi-ku, Fukuoka, 813-8529, Japan
*Present address: Department of Pharmacology and Biological Chemistry, Mount Sinai School of Medicine, New York, NY 10029, USA
†Author for correspondence (e-mail: h-okamoto@aist.go.jp)

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Summary

Early neural patterning along the anteroposterior (AP) axis appears to involve a number of signal transducing pathways, but the precise role of each of these pathways for AP patterning and how they are integrated with signals that govern neural induction step is not well understood. We investigate the nature of Fgf response element (FRE) in a posterior neural gene, *Xcad3* (*Xenopus caudal* homologue) that plays a crucial role of posterior neural development. We provide evidence that FREs of *Xcad3* are widely dispersed in its intronic sequence and that these multiple FREs comprise Ets-binding and Tcf/Lef-binding motifs that lie in juxtaposition. Functional and physical analyses indicate that signaling pathways of Fgf, Bmp and Wnt are integrated on these FREs to regulate the expression of *Xcad3* in the posterior neural tube through positively acting Ets and Sox family transcription factors and negatively acting Tcf family transcription factor(s).

Key words: Fgf, Xcad3, Ets, Tcf/Lef, Sox, Neural patterning, *Xenopus*

Introduction

Development of the vertebrate nervous system is initiated by demarcation of the neurogenic region from the surrounding epidermal region in the embryonic ectoderm (neural induction), which is followed by generation of anteroposterior (AP) pattern within the neurogenic region (neural patterning). The induced new ectoderm tissue exhibits initially anterior neural character (forebrain); subsequent patterning step then specify more posterior neural character (midbrain, hindbrain, spinal cord) (Nieuwkoop, 1952; Slack and Tannahill, 1992). Recent molecular studies have revealed that a number of signal transducing pathways are involved in neural induction, either positively or negatively, in amphibian and amniote embryos (Wilson and Hemmati-Brivanlou, 1997; Wilson and Edlund, 2001). These include Fgf, Wnt and Bmp signaling pathways. Notably, the neural pattering step seems also to include signaling by Fgf (Cox and Hemmati-Brivanlou, 1995; Hongo et al., 1999; Kengaku and Okamoto, 1995; Lamb and Harland, 1995) and Wnt (Kiecker and Niehrs, 2001; McGrew et al., 1995), in addition to that by retinoic acid (Blumberg et al., 1997; Kolm et al., 1997). Classic experiments have predicted that patterning signals act in a gradient, with higher signaling level conferring more posterior neural character to the anteriorly induced tissue (Nieuwkoop, 1952; Slack and Tannahill, 1992).

Among the candidate signaling molecules for neural induction and patterning, Fgf is of particular interest. Fgf can change the developmental fate of *Xenopus* ectoderm cells in culture from epidermal to neural (Kengaku and Okamoto, 1993), and induce these cells to express position-specific neural markers along AP axis in a dose-dependent manner, with higher doses eliciting more posterior neural marker genes (Kengaku and Okamoto, 1995). Furthermore, loss-of-function experiments have shown that Fgf signaling is required for both anterior (Hongo et al., 1999) and posterior (Holowacz and Sokol, 1999; Pownall et al., 1996; Ribisi et al., 2000) neural development, as judged by expression of positional marker genes. However, a recent report indicates that dose-dependent Wnt signaling is both necessary and sufficient for AP neural patterning (Kiecker and Niehrs, 2001), although another report indicates that Wnt signaling posteriorizes neural tissue through elevating the level of Fgf signaling (Domingos et al., 2001). Thus, the precise role of each of these signaling pathways for the establishment of AP neural pattern and how they are integrated with signaling in the preceding neural induction step is still not clear.

To address these questions, we may need not only loss- or gain-of-function experiments, but also an approach to directly identify *cis*-acting sequence elements in positional marker genes, which respond to the neural patterning signals. In this study, we investigated the nature of the Fgf response element (FRE) in a posterior marker gene, *Xcad3* (Northrop and Kimelman, 1994). *Xcad3*, a *Xenopus caudal* homologue encoding a homeodomain transcription factor, lies downstream of Fgf signaling and functions as an upstream activator of several Hox genes that regulate posterior embryonic development (Isaacs et al., 1998; Northrop and Kimelman, 1994; Pownall et al., 1996), as has been implicated for some

members of the *caudal* gene family of other vertebrate species (Cdx genes in mammals and chicken) (Deschamps et al., 1999).

We have isolated an *Xcad3* genomic clone containing regulatory elements that drive *Xcad3* expression in the posterior neural tube in response to Fgf signaling. We provide evidence that FREs of *Xcad3* are widely dispersed in the first intron and we demonstrate that these multiple FREs comprise Ets-binding and Tcf/Lef-binding motifs (EBMs and TLBMs) that lie in juxtaposition. Functional analysis shows that Ets family transcription factors are indeed involved in the Fgf response of *Xcad3* activation. This indicates that *Xcad3* is directly targeted by Fgf signaling, as Ets proteins are nuclear effectors of Fgf/Ras/mitogen-activated kinase (Mapk) pathway (Wasylyk et al., 1998). By contrast, XTcf3, a nuclear effector of Wnt/β-catenin pathway (Molenaar et al., 1996), functions as a repressor of *Xcad3* (Nusse, 1999). Furthermore, Sox2, a Sry-related transcription factor that shares the cognate DNA-binding motif with Tcf/Lef family members (Kamachi et al., 2000) is shown to cooperate with Ets proteins, possibly by competing with XTcf3 for TLBMs in the composite FREs. Direct interaction of these proteins with some EBMs and TLBMs were demonstrated in gel mobility shift assays. Sox2 is de-repressed in the neurogenic region by Bmp antagonists during the neural induction step (Mizuseki et al., 1998a). Our results thus indicate that signaling pathways of Bmp, Fgf and Wnt are integrated on the FREs to regulate the expression of *Xcad3* in the posterior neural tube during neural patterning.

Materials and methods

Cloning of *Xcad3* genomic DNA

Xenopus genomic library (Stratagene) was screened with a probe containing 383 bp (from the translation start site to 383) cDNA (Northrop and Kimelman, 1994) in *Xcad3*, which was prepared by PCR from cDNA synthesized from *Xenopus* neurula stage mRNA. The transcription initiation site was determined by 5′ RACE using the 5′-Full RACE Core Set (TaKaRa).

Plasmid construction

The 5′ flanking and first intron of *Xcad3* were subcloned into a luciferase reporter plasmid pGL3-Basic Vector (Promega), separately or in combination. The 5′ flanking sequence was inserted upstream of the luciferase coding sequence, although the first intron sequence was inserted downstream as follows. An *Eco*RI fragment of a genomic clone (–1741 to +389) was subcloned into the *Eco*RI site of pBluscript II SK- (Stratagene); sequence –1741 to +174 (one nucleotide upstream of the translation start site) was amplified by PCR and cloned into blunted *Nco*I site of pGL3-Basic Vector. *Sac*I (in the phage arm)-*Eco*RI fragment (–7000 to –1741) was then cloned into the *Sac*I-*Eco*RI site of pGL3-Basic that contained 5′ fragment –1741 to +175. The first intron sequence was amplified by PCR and cloned into the *Bam*HI site of pGL3-Basic. To generate chimeric constructs with SV40 sequences, pGL3-Promoter Vector that contains SV40 promoter sequence upstream of luciferase sequence, or pGL3-Enhancer Vector that contains SV40 enhancer sequence downstream of it was used instead of pGL3-Basic Vector. To generate constructs containing 5′ flanking and intronic sequence deletion, respective PCR fragments were cloned into pGL3-Basic as above. For a GFP reporter plasmid, the luciferase-coding region was removed from pGL3 vector sequence by *Nco*I-*Xba*I digestion and replaced by a *Nco*I-*Xba*I fragment from pEGFP-N3 (Clontech), containing the EGFP-coding sequence. Mutations of EBMs and TLBMs were introduced by Ex-Site mutagenesis kit (Stratagene). The entire or deleted coding sequences of XEts1 (Meyer et al., 1997), XER81 (Chen et al., 1999; Munchberg and Steinbeisser, 1999), human ELK1 (Chen et al., 1999), XTcf3 (Molenaar et al., 1996), XLef1 (Molenaar et al., 1998) and β-catenin (Molenaar et al., 1996) were amplified by PCR and subcloned into pSP64T. Vp16-XTcf3 was as published (Kim et al., 2000). Sox2-EnR was made by in-frame C-terminal fusion of the *Drosophila engrailed* repressor region (Conlon et al., 1996) to Sox2. Sox2, Sox2 BD(–) and SoxD BD(–) were kindly provided by Y. Sasai (Kishi et al., 2000; Mizuseki et al., 1998a; Mizuseki et al., 1998b).

Microinjection and transgenesis

Microinjection of reporter and internal standard plasmids with or without synthetic mRNA of various transcription factors was performed as previously described (Hongo et al., 1999). Injected plasmids were adjusted to the same on a molar base and they were injected at 3×10^{-18} moles in 1.6 nl/blastomere of eight-cell stage *Xenopus* embryos. Transgenic embryos were generated as described (Kroll and Amaya, 1996).

Microculture and quantitative RT-PCR

Injected or uninjected *Xenopus* gastrula embryos were used. Methods for culturing ectoderm cells were essentially as previously described (Kengaku and Okamoto, 1993). RNA was extracted from 20 cultures for each experimental point and subjected to quantitative RT-PCR as previously described (Hongo et al., 1999; Kengaku and Okamoto, 1995).

Luciferase assay

The luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega), in which firefly luciferase in pGL3 was used for the reporter gene assay, whereas Renilla luciferase in the internal standard plasmid pRL-CMV was used for normalization. After a group of injected embryos or cultures were incubated up to the desired stage, they were homogenized in Passive Lysis buffer (Promega). For the embryonic cell culture assay, half the culture medium in each culture well was replaced by 2×Passive Lysis buffer and 20 cultures were collected and homogenized for each experimental point. The lysate was centrifuged at 18,000 ***g*** for 1 minute at 4°C. The clear supernatant was assayed with firefly luciferase substrate and Renilla luciferase substrate separately to avoid possible interference. Each luciferase activity was measured three times, and the mean value was used. All of the injection experiments were carried out at least three times and gave reproducible results. One representative experiment was shown for each figure.

Gel mobility shift assay

V5-epitope-tagged XTcf3, Sox2 and XEts1 proteins were made by in vitro translation with a rabbit reticulocyte lysate (Promega), and gel mobility shift assays were performed as described (Huang et al., 1995). DNA fragments used as probes were 3′ end-labeled with digoxygenin-11-ddUTP according to the manufacturer's recommendations (Roche Diagnostics; DIG Gel Shift Kit). Supershifts were generated by adding 1 μl of monoclonal antibody directed against V5 epitope (Invitrogen). DNA-protein complexes were separated by electrophoresis through 3.5% polyacrylamide gel containing 0.5×TBE and 2.5% glycerol. Gels were further processed according to the manufacturer's recommendation (Roche Diagnostics; DIG Gel Shift Kit).

Results

5′ flanking and intronic sequences direct Fgf-dependent *Xcad3* expression in the posterior neural tube

By screening a *Xenopus* genomic library with an *Xcad3* cDNA probe, we obtained four overlapping *Xcad3* clones that

encompassed a 5′ flanking sequence (about 7000 bp), full coding sequence and a 3′ flanking sequence (about 13,000 bp), collectively. A comparison of the genomic sequence with cDNA sequence (Northrop and Kimelman, 1994) showed that *Xcad3* gene had two introns within the coding sequence, 3946 bp (intron1) and 291 bp (intron2) in length, respectively (Fig. 1A). The overall structures of the *Xcad3* gene was similar to that of human and mouse Cdx genes, as inferred from draft and finished sequences deposited in NCBI database.

We asked whether the *Xcad3* genomic clone contains regulatory elements that drive *Xcad3* expression in the posterior neural tube (spinal cord) under the control of Fgf signaling. For this, we subcloned the 5′ flanking region and intron1 into a luciferase reporter plasmid pGL3, separately or in combination (Fig. 1B). These constructs were injected into the prospective anterior brain or posterior neural tube region of eight-cell stage embryos (Hirose and Jacobson, 1979) (AB or PNT in Fig. 1B). We found that robust luciferase activity was induced only when the reporter construct contained both 5′ flanking and intron1 sequences, and injected into the PNT site. Reporter constructs containing either 5′ flanking or intron1 sequence alone were not as effective as the full construct irrespective of the site of injection. In several series of experiments, the spatial specificity (PNT versus AB) in the reporter activities reached more than 10-fold reproducibly.

To locate regulatory elements in the 5′ flanking sequence, deletion analysis was carried out using the luciferase assay system. We found that a truncation down to –185 (relative to the transcription initiation site) did not largely affect the spatial specificity and the extent of luciferase expression (details not shown, but see Fig. 1D for an example).

We then tested the ability of the 5′ immediate upstream sequence and intron1 to regulate spatiotemporal expression pattern of *Xcad3*. For this we replaced luciferase with green fluorescent protein (GFP) as a reporter, and generated transgenic embryos carrying the GFP construct (–185/GFP/intron1 as depicted in Fig. 1C). GFP expression was first detected at stage 11 in the marginal zone, the prospective mesoderm region. During neurula and tail bud stages, GFP was expressed primarily in the posterior of the developing neural tube (Fig. 1C). This spatiotemporal expression pattern of GFP was consistent with that of the endogenous *Xcad3* as determined by in situ hybridization, except for a stripe of GFP expression above the eye (Northrop and Kimelman, 1994). The ectopic expression may reflect a high level of Fgf signals at the midbrain/hindbrain boundary (Christen and Slack, 1997; Tannahill et al., 1992). It is likely that additional elements would be necessary to suppress GFP expression in this region.

We further asked whether the reporter activity induced by the 5′ upstream elements and intron1 of *Xcad3* was dependent on Fgf signaling. For this, mRNA encoding a dominant-negative *Xenopus* Fgf receptor type 4a (ΔXFgfR4a) (Hongo et al., 1999) were co-injected into the PNT site at the eight-cell stage. We found that overexpression of ΔXFgfR4a caused a strong suppression of luciferase activity induced by –7000/LUC/intron1 or –185/LUC/intron1 construct (Fig. 1D). Although the extent of suppression varied somewhat in several series of experiments, it averaged more than 80%.

Collectively, our results indicate that regulatory elements present in 5′ upstream and intron1 sequences are sufficient to drive *Xcad3* expression in the posterior neural tube and they include FREs, which are indispensable for *Xcad3* expression.

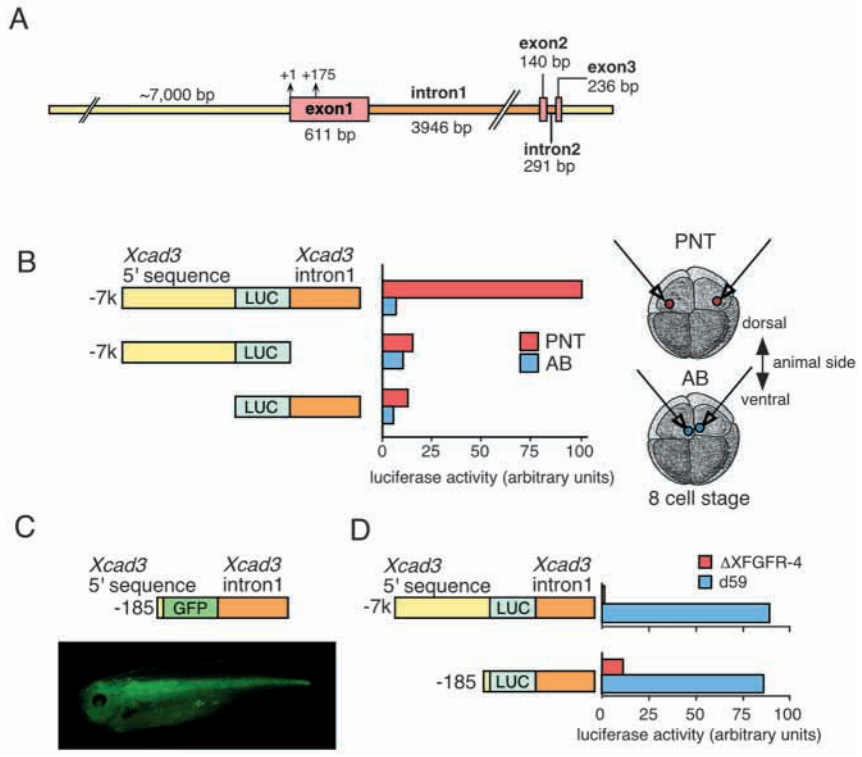

Fig. 1. Structural and functional analysis of the *Xenopus Xcad3*. (A) The *Xcad3* genomic clone. Three exons and two introns are depicted with their respective nucleotide length together with 5′ and 3′ flanking regions. The transcription and translation initiation sites are numbered as +1 and +175, respectively. (B) The regulatory function of the genomic *Xcad3* sequences. The 5′ flanking (–7000 to +174) and intron 1 sequences were inserted into a firefly luciferase (LUC) reporter plasmid pGL3, as illustrated left of the histogram. These *Xcad3/LUC* constructs were injected into eight-cell stage embryos together with an internal standard plasmid pRL-CMV that contained Renilla luciferase coding sequence. Injection sites are depicted right of the histogram as PNT and AB. Regions around these sites give rise to the posterior neural tube and anterior brain, respectively. Luciferase activities were measured at stage 23 and *Xcad3/LUC* reporter activities normalized to the pRL-CMV internal standard activities were presented in the histogram with arbitrary units. (C) Transgenic embryo carrying *Xcad3/GFP* construct. The reporter construct used for transgenesis is indicated on top. The transgenic embryo shown was photographed at stage 37. (D) Involvement of Fgf signaling in enhancing function of the 5′ flanking and intron1 sequences. *Xcad3/LUC* reporter constructs indicated left of the histogram were injected into the PNT site together with mRNA (100 pg/blastomere) encoding ΔXFgf-4a or d59 (a control inactive version of ΔXFgfR-4a). The d59 lacks a stretch of 59 amino acids that is required for dimerization with a wild-type receptor subunit (Amaya et al., 1991). Luciferase activities were analyzed and presented as in B.

High-dose-dependent FREs are present in intron1

Fgf can induce *Xenopus* gastrula ectoderm cells in culture to express position-specific neural marker genes along the anteroposterior axis in a dose-dependent manner; with lower doses eliciting more anterior marker genes such as *XeNK2* or *En2* and higher doses more posterior marker genes such as *XlHbox1* (*Hoxc6*) or *XlHbox6* (*Hoxb9*) (Kengaku and Okamoto, 1995). Indeed endogenous *Xcad3* was activated in considerably higher Fgf dose range than *En2*, when examined in the embryonic cell culture assay (Fig. 2A,B).

We then asked whether FREs that were supposed to be located within the 5′ flanking sequence and/or intron1 of *Xcad3* exhibited such a high dose dependence on Fgf. Experimental design is shown in Fig. 2C. It was found that the Fgf dose-response profile for luciferase activity of –7000/LUC/intron1 construct coincided very well with that for transcriptional activity of endogenous *Xcad3* (Fig. 2D). Similar results were obtained with reporter constructs containing truncated 5′ upstream sequences such as –185/LUC/intron1, but those containing either 5′ sequences or intron1 alone failed to respond to bFgf (not shown).

Where, then, are the FREs of *Xcad3*, in the 5′ upstream sequence or in intron1? To answer this question, we prepared chimeric constructs in which the 5′ sequence and intron1 were replaced with SV40 promoter and enhancer sequences, respectively, as depicted in Fig. 2E,F. These SV40 elements were used to enhance reporter activities, as 5′ sequence or intron1 alone could not induce sufficient reporter activities for quantitative analysis, as shown in Fig. 1B. We found that a chimeric construct containing intron1 (SV40 promoter/Luc/

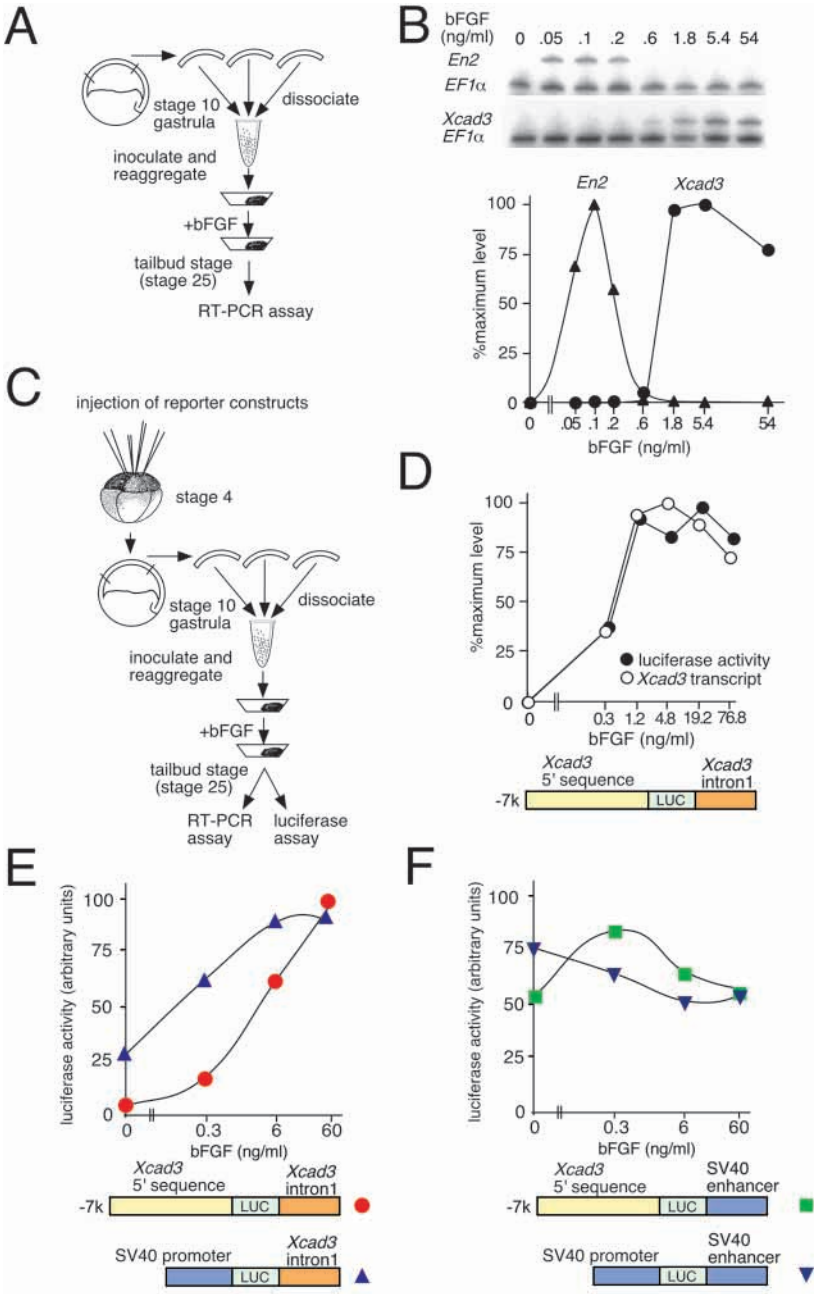

Fig. 2. Analysis of Fgf response element (FRE) of *Xcad3*. (A) Experimental design for the embryonic cell culture assay used in B. Ectodermal tissues were isolated from stage 10 gastrula embryos. The dissociated cells were inoculated into microculture wells at 200 cells/well. After completion of reaggregation by brief centrifugation, cells were cultured in the presence of increasing concentrations of bFgf until control embryos reached stage 23. The transcriptional levels of two position-specific neural markers were analyzed by RT-PCR (Kengaku and Okamoto, 1995). (B) High-dose-dependent activation by Fgf of endogenous *Xcad3*. Autoradiographs are shown of RT-PCR products of the transcripts from *En2*, an anterior neural marker gene and *Xcad3*, both of which were co-amplified with *EF1α* transcript, an internal standard (upper panels). Each RT-PCR product was quantified by a laser image analyzer and values for *En2* (▲) and *Xcad3* (●) transcripts normalized to *EF1α* transcript are presented as percentages of the respective maximum value and plotted against bFgf dose (graph). (C) Experimental design for the embryonic cell culture assay used in D. *Xcad3/LUC* reporter and pRL-CMV plasmids were coinjected into four animal blastomeres of eight-cell stage embryos. When they reached stage 10, ectodermal tissues were isolated and processed as in A. To compare directly Fgf dose-dependence of *Xcad3/LUC* reporter with that of endogenous *Xcad3*, two parallel sets of cultures were prepared; one was assayed for luciferase activity, while the other was assayed for transcriptional levels of endogenous *Xcad3*. (D) Comparison of the Fgf dose-dependence profiles for a *Xcad3/LUC* reporter and endogenous *Xcad3*. Eight-cell stage embryos were injected with a *Xcad3/LUC* reporter depicted below the graph and an internal standard plasmid pRL-CMV and processed as described in C. Normalized *Xcad3/LUC* reporter activities are presented as percentages of the maximum value and plotted against bFgf dose (●). Transcript levels of endogenous *Xcad3* was assayed and plotted as in B (○). (E,F) Presence of FRE in the intron 1. Chimeric constructs injected are indicated below each graph: they contained either *Xcad3* intron1 (E) or SV40 enhancer sequence (F). Reporter activities of these constructs were analyzed as in D and presented in arbitrary units. Note that inclusion of intron1 sequence in reporter constructs is essential for dose-dependent response to Fgf.

intron1) exhibited a dose-dependent response to Fgf (Fig. 2E, ▲), but any other constructs examined including –7000/LUC/SV40 enhancer, SV40 promoter/LUC/SV40 enhancer (Fig. 2F, ■, ▼), or –185/LUC/SV40 enhancer (not shown) did not show such dose-dependence on Fgf. However, the Fgf dose-response profile of the active construct (SV40 promoter/LUC/intron1) did not coincide well with that of the intact construct (Fig. 2E; ●, –7000/LUC/intron1) in this and other series of experiments. It is likely that the FREs of *Xcad3* are primarily located within the intron1 but their interaction with 5′ upstream elements through specific transcription factors is required to mediate the proper dose-dependent response of *Xcad3* to Fgf.

FREs comprise Ets-binding and Tcf/Lef-binding motifs

To identify the FREs in the intron1, deletion analysis was carried out using the embryonic cell culture assay. A control construct containing the full-length intron1 exhibited at least 20-fold activation of the reporter activity by the addition of bFgf as exemplified in Fig. 3A(i). Several series of experiments showed that the 5′-most 1200 bp and 3′-most 600 bp sequences were dispensable (not shown). Further serial deletion of 1100 bp from the 5′ side resulted in progressive loss of Fgf responsiveness [Fig. 3A(ii-iv)]. Similar gradual change was reproducibly observed by deleting this region consecutively, indicating that multiple FREs are distributed within it (domain 1 in Fig. 3A). Notably, the sequence 3′ to the domain 1 still retained Fgf responsiveness that amounted to about 5-fold activation as exemplified in Fig. 3A(iv). Indeed, several series of deletion experiments from the 3′ side [Fig. 3A(v), (vi), for example] showed that there was another domain that contained FREs (domain 2 in Fig. 3A). Progressive loss of Fgf responsiveness by serial deletions within the domain 2 was exemplified in Fig. 3B (see below for more detail). These results indicate that FREs are not localized in a narrow region within intron 1 but rather widely dispersed throughout it, conferring the full Fgf responsiveness to intron 1 in a coordinated manner.

To search for candidate DNA motifs that constitute FREs, sequence analysis was carried out on intron 1. We noticed that a number of Ets-binding motifs (EBMs) were present throughout intron1 (Fig. 3A, blue triangles). The EBM (consensus sequence A/C GGA A/T) (Sharrocks et al., 1997) is known to serve as the binding site for the Ets family of transcription factors that are nuclear effectors of the Fgf/Ras/Mapk pathway (Wasylyk et al., 1998). In *Xenopus* embryos, this pathway represents a major transducing pathway from Fgf ligand to its nuclear targets (LaBonne and Whitman, 1997; MacNicol et al., 1993; Weinstein et al., 1998; Whitman and Melton, 1992). Interestingly, many of the EBMs in domains 1 and 2 were in the proximity of Tcf/Lef binding motifs (TLBM; consensus sequence CTTTGA/TA/T) (van de Wetering et al., 1997), as illustrated in Fig. 3A (red triangles) and Fig. 4A. The TLBM serves as the binding site for the Tcf/Lef family of transcription factors, nuclear targets of the Wnt/β-catenin pathway in *Xenopus* embryos (Molenaar et al., 1996).

Consecutive deletion targeting some of TLBMs and EBMs in domain 2 resulted in a progressive reduction of Fgf responsiveness (Fig. 3B). We then asked whether these EBMs and TLBMs in intron 1 indeed constitute FREs that mediate the transcriptional response to Fgf. For this, we introduced various combinations of mutations into EBMs or TLBMs in domain 2* that included all possible EBMs and TLBMs around domain 2 (Fig. 3A, Fig. 4A), and examined Fgf dose-response profiles of these mutated constructs. Among an extensive series of experiments, typical ones are presented in Fig. 4B,C. Introduction of mutations into an increasing number of EBMs resulted in a progressive loss of Fgf responsiveness, reaching nearly complete elimination by mutagenesis of all possible

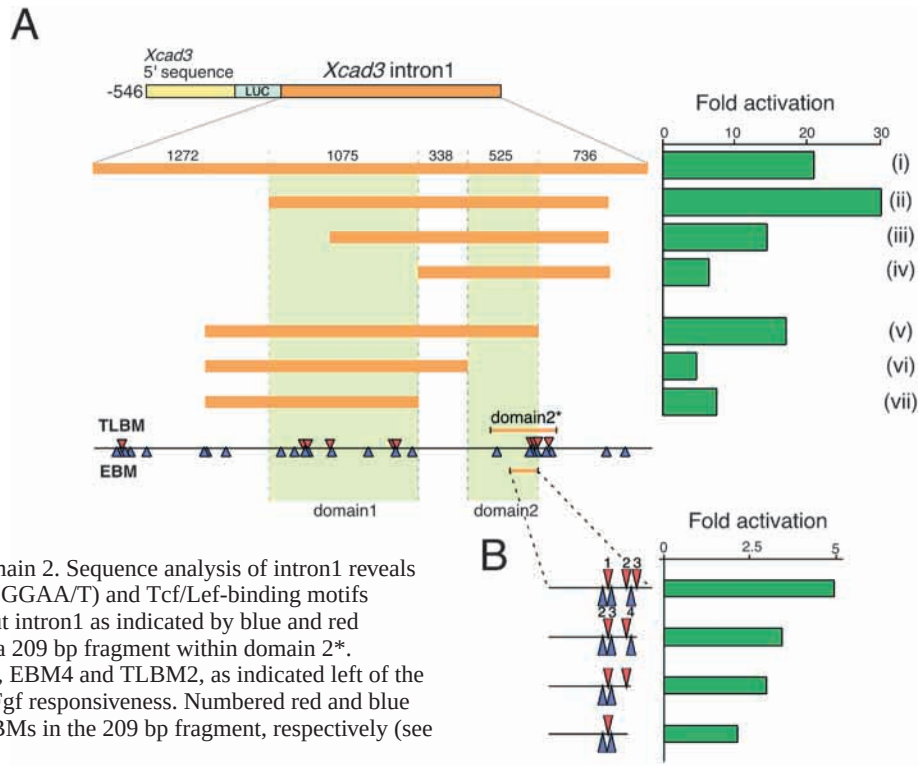

Fig. 3. Identification of multiple FREs within the intron 1. (A) Deletion and sequence analysis of the intron 1. Reporter constructs examined contain intron 1 sequences that are deleted to various extents as depicted left of the histogram. Reporter activities of these constructs were analyzed as in Fig. 2, except that ectoderm cells were cultured in the absence or presence (6 ng/ml) of bFgf. The ratio of the luciferase activity obtained with bFgf to that obtained without bFgf is taken as a measure of Fgf responsiveness and presented as a value of fold induction in the histograms. Numbers on full-length intron 1 indicate nucleotide length of respective segments. Multiple FREs appear to be distributed within domain1 and domain 2 (light green) of intron 1. Domain 2*, as defined in the text, is also indicated close to domain 2. Sequence analysis of intron1 reveals that a number of Ets-binding motifs (EBM; A/CGGAA/T) and Tcf/Lef-binding motifs (TLBM; CTTTGA/TA/T) are present throughout intron1 as indicated by blue and red triangles, respectively. (B) Deletion analysis of a 209 bp fragment within domain 2*. Consecutive deletion of binding motifs TLBM3, EBM4 and TLBM2, as indicated left of the histogram results in a progressive reduction of Fgf responsiveness. Numbered red and blue triangles point to the position of TLBMs and EBMs in the 209 bp fragment, respectively (see Fig. 4A for precise location).

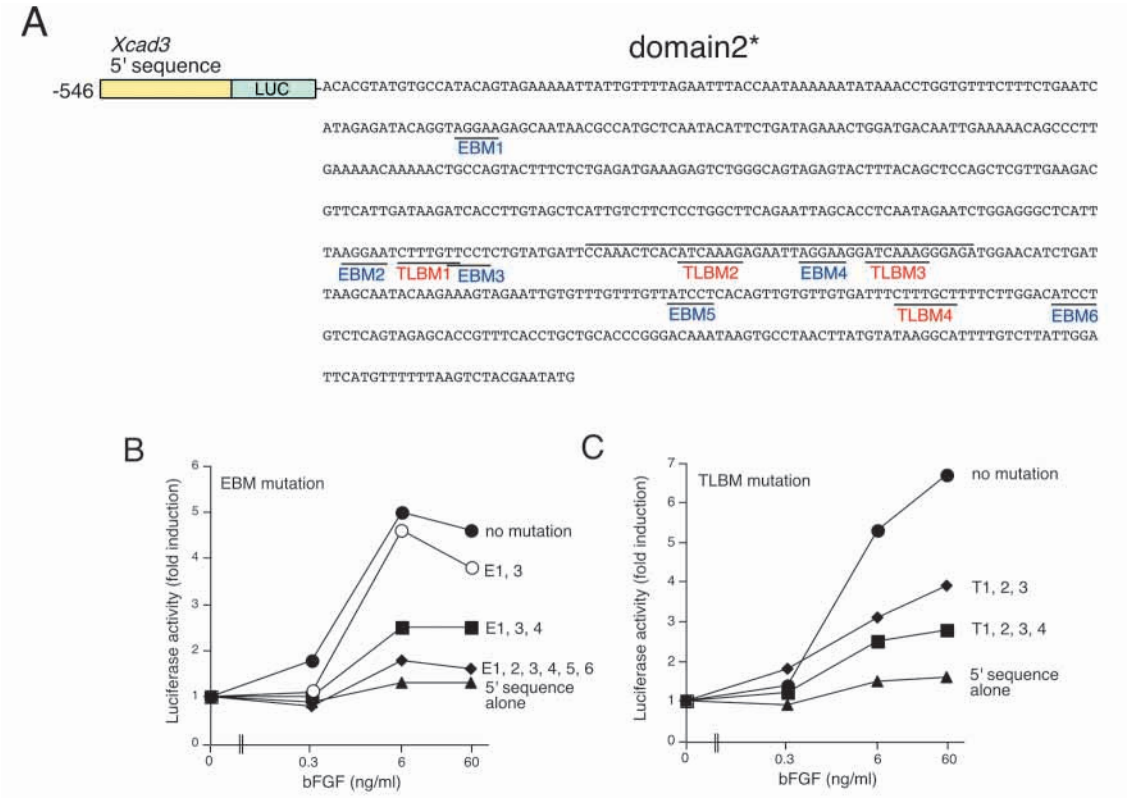

Fig. 4. EBMs and TLBMs as sub-elements of FREs. (A) Nucleotide sequence of the domain 2* region of *Xcad3* intron 1. EBMs and TLBMs are marked and numbered. (B,C) Mutational analysis of EBMs (B) and TLBMs (C). Reporter constructs examined contain the domain 2* fragments that carry various combinations of mutations in EBMs or TLBMs, as indicated to the right of each graph (E1, 3 means that EBM1 and EBM3 are mutated). Mutations were introduced as follows: GAA to agA in E1, 2 and 4; TCC to ctC in E3 and 5; ATCCT to gTCga in E6; TTTGT to Tcgag in T1; TCAAA to TagAc in T2; TCAAAGG to Tgtcgac in T3; CTTTG to gaaTt in T4. Reporter activities of mutated constructs were analyzed as in Fig. 3, except that ectoderm cells were cultured in the absence or presence of increasing concentrations of bFgf.

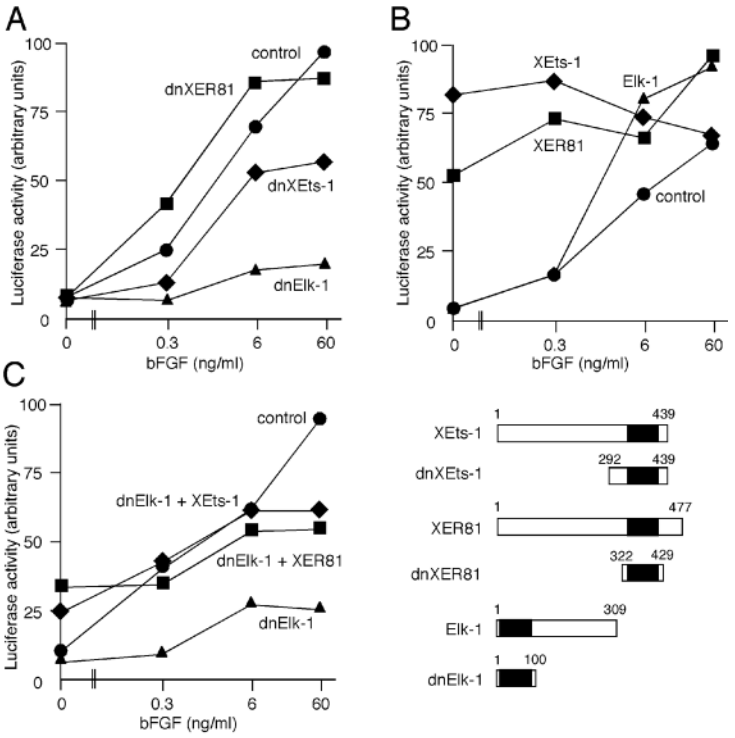

Fig. 5. Involvement of Ets transcription factors as activators in the Fgf response of *Xcad3*. Structural features of Ets proteins and their derivatives examined are illustrated at the bottom right. Black boxes represent the DNA-binding ets domains. Synthetic mRNAs encoding these proteins were coinjected with –546/LUC/intron1 and pRL-CMV into two animal blastomeres (either left or right side) of eight-cell stage embryos, which were then processed as in Fig. 2C. Reporter activity was analyzed as in Fig. 2D and presented in arbitrary units. (A) Effects of dominant-negative Ets proteins on the Fgf response of *Xcad3/LUC*. The injected amount was 32 pg/blastomere for dnElk1 (▲), dnXEts1 (◆), dnXER81 (■) and EnR (●) mRNA (control). (B) Effects of wild type Ets proteins on the Fgf response of *Xcad3/LUC*. The injected amounts are 30 pg/blastomere for Elk1 (▲), XEts1 (◆), XER81 (■) and EnR (●) mRNA. Note that XEts1 and XER81 activate the reporter gene more efficiently than Elk1. (C) Reversal of dnElk1 induced suppression of Fgf response by XEts1 and XER81. The injected amounts were 24 pg, 48 pg, 72 pg and 96 pg/blastomere for dnElk1, XEts1, XER81 and EnR mRNA. The total amount of injected mRNA was adjusted by adding neutral EnR mRNA. Note that the suppression of reporter gene expression induced by dnElk1 (▲) is reversed by the addition of XEts1 (◆) or XER81 (■).

EBMs in domain 2* (Fig. 4B). Similar gradual loss of Fgf responsiveness was obtained by mutating an increasing number of TLBMs (Fig. 4C). Our results indicate that both EBMs and TLBMs are required for conferring Fgf responsiveness to domain 2*. By extrapolating from these results, one might expect that EBMs and TLBMs in domain 1, which lie in juxtaposition, are also required for its responsiveness to Fgf. Although this issue has yet to be confirmed, our data strongly suggest that EBMs and TLBMs are components of FREs in intron 1.

Ets transcription factors mediate the response of *Xcad3* to Fgf

We next explored the transcription factors that interact with the FREs of *Xcad3* to mediate the Fgf response. Obvious candidates are Ets family and Tcf/Lef family proteins. Notably, transcripts of some of the Ets genes including *Ets1*, *Ets2* (Meyer et al., 1997) and *ER81* (Chen et al., 1999; Munchberg and Steinbeisser, 1999) as well as that of *XTcf3* (Molenaar et al., 1996) are expressed in early *Xenopus* embryos. These transcription factors would act cooperatively through binding to respective motifs in the composite FREs.

We first assessed the functional role of Ets proteins, as they are potential nuclear effectors of the Fgf/Ras/Mapk signal transducing pathway (Wasylyk et al., 1998). A mRNA encoding a dominant-negative form of *Xenopus* Ets1 (dnXEts1), ER81 (dnXER81) or human Elk1 (dnElk1) was co-injected with a reporter construct. These dominant-negative constructs lack the N-terminal (dnXEts1, dnXER81) or C-terminal (dnElk1) regions of the respective wild-type proteins, which include the activation domain (Fig. 5, bottom right). These mutants thus mainly comprise the DNA-binding ets domain, thereby potentially competing with endogenous Ets proteins for the EBMs (Wasylyk et al., 1994). Overexpression of dnElk1 and dnEts1 to a lesser extent caused a suppression of the response of the reporter construct to Fgf, while that of dnXER81 did not suppress the response (Fig. 5A). Increasing the amount of co-injected mRNA suppressed more effectively the Fgf response for dnEts1, but not for dnXER81 (not shown).

These results show that functional activity of endogenous Ets proteins are required for the Fgf response of *Xcad3*. However, they do not necessarily imply that the Elk1-type and Ets1-type but not ER81-type proteins are responsible for the response, as Ets proteins have overlapping DNA binding specificities (Wasylyk et al., 1994). Indeed, when the effects of overexpression of the wild type Ets proteins were examined, XEts1 and XER81 were shown to more effectively activate the reporter gene than Elk1 (Fig. 5B). Furthermore, the wild-type XEts1 and XER81 could reverse the suppression of the Fgf response caused by dnElk1 (Fig. 5C) or dnXEts1 (not shown).

Collectively, our results indicate that some of the Ets transcription factors are involved as activators in the response of *Xcad3* to Fgf. It is highly likely that these Ets proteins bind to EBMs in composite FREs in intron 1, and that Fgf signaling enhances their ability to activate transcription by phosphorylating them through Mapk.

XTcf3 represses Fgf response of *Xcad3*

We next assessed the functional role of XTcf3, a nuclear target of the Wnt/β-catenin pathway, using a dominant-negative XTcf3 (dnXTcf3) construct. dnXTcf3 lacks the N-terminal region that is required for binding of β-catenin, a co-activator of XTcf3, but retains the ability to bind its cognate DNA motif, thereby abrogating transcriptional activation by the endogenous β-catenin-XTcf3 complex (Molenaar et al., 1996). In embryonic cell culture assays, overexpression of dnXTcf3 resulted in a suppression of the response to Fgf of a reporter construct in a dose-dependent manner (Fig. 6A). Surprisingly, however, overexpression of wild-type XTcf3 also caused a profound suppression of the Fgf response instead of an activation (Fig. 6B). This was unexpected, because Wnt signaling was suggested to be involved in activation of

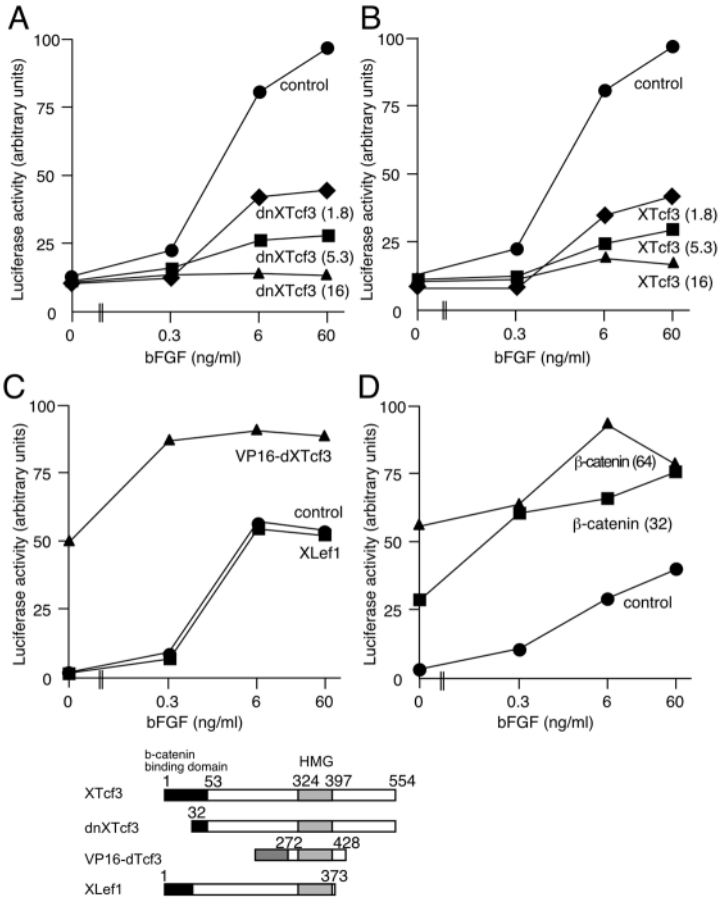

Fig. 6. Involvement of XTcf3 as a repressor in the Fgf response of *Xcad3*. Structural features of XTcf3, its derivatives and XLef1 examined are illustrated at the bottom. β-catenin binding domains, VP16 activation domain and DNA-binding HMG domains are marked. Experimental procedures are as described in Fig. 5. (A) Effects of dominant-negative XTcf3 on the Fgf response of *Xcad3/LUC*. The injected amounts were 1.8 (◆), 5.3 (■) or 16.0 (▲) pg dnXTcf3 mRNA/blastomere. (B) Effects of wild-type XTcf3 on the Fgf response of *Xcad3/LUC*. The injected amounts were 1.8 (◆), 5.3 (■) or 16.0 (▲) pg XTcf3 mRNA/blastomere. (C) Effects of XLef1 and VP16-dXTcf3 on the Fgf response of *Xcad3/LUC*. The injected amount was 6 pg/blastomere for XLef1 (■), VP16-dXTcf3 (▲) and EnR mRNA (●). VP16-dXTcf3 enhances the Fgf response of the reporter construct (–546/LUC/intron1), but XLef1 does not. (D) Effects of β-catenin on the Fgf response of *Xcad3/LUC*. The injected amounts were 32 (■) or 64 (▲) pg β-catenin mRNA/blastomere.

posterior neural genes (Kiecker and Niehrs, 2001; McGrew et al., 1995) and we had anticipated that XTcf3 functioned as an activator of *Xcad3* by binding β-catenin. It should be noted, however, that XTcf3 has also been shown to function as a transcriptional repressor instead of an activator by binding co-repressors such as Groucho (Roose et al., 1998) or XCtBP (Brannon et al., 1999) in place of β-catenin. Overexpression of XLef1, another Tcf/Lef family member that lacks the repressing function of XTcf3, but retains the activating function by binding β-catenin (Brannon et al., 1999; Molenaar et al., 1998) had no effect on the Fgf response (Fig. 6C). It is possible that the endogenous pool of β-catenin is considerably smaller compared with those of XCtBP, Groucho or other co-repressors in *Xenopus* ectoderm cells, and XTcf3 (VP16-dXTcf3) may act primarily as a repressor in these cells. Indeed, overexpression of β-catenin itself or a mutant construct in which the VP16 activation domain was fused to truncated XTcf3 counteracted the repressing action of endogenous XTcf3 (Kim et al., 2000): in embryonic cell culture assays, they induced robust luciferase activity (Fig. 6C,D). By contrast, overexpression of Wnt8 protein, which would facilitate endogenous β-catenin to complex with XTcf3 (Domingos et al., 2001; Molenaar et al., 1996) did not affect the Fgf response (not shown). Collectively, our results indicate that XTcf3 functions primarily as a repressor of *Xcad3*. This raises the possibility that Ets proteins overcome this repression by cooperating with other transcription factors that bind to TLBMs in place of XTcf3.

Sox2 is involved as the co-activator in the Fgf response of *Xcad3*

It has been shown that signaling mediated by Sox2, a Sry-related transcription factor, is required for the expression of *Hoxb9*, a downstream target of *Xcad3* (Isaacs et al., 1998), in the posterior neural tube (Kishi et al., 2000). Notably, Sox2 is a member of the Sox family proteins, which share a DNA-binding high-mobility group (HMG) domain with Tcf/Lef family proteins (Kamachi et al., 2000). The cognate motif of Sox2 (CA/TTTGTT) (Kamachi et al., 2000) is accordingly very similar to that of Tcf/Lef proteins (CTTTGA/TA/T) (van de Wetering et al., 1997). This raises the possibility that Sox2 competes with XTcf3 for TLBMs to activate *Xcad3* in cooperation with Ets proteins, which leads to upregulation of *Hoxb9*. Indeed, a combination of Sox2 and bFgf was shown to activate posterior neural genes, including *Hoxb9* in the animal cap assay (Mizuseki et al., 1998a).

Possible involvement of Sox2 in the Fgf response of *Xcad3* was examined in the embryonic cell culture assay using a dominant-negative version of Sox2 in which the engrailed repressor domain was fused with Sox2 (Sox2-EnR) (Conlon et al., 1996). Overexpression of Sox2-EnR resulted in a suppression of the Fgf response of a reporter construct containing the full-length of intron 1 (Fig. 7A). By contrast, overexpression of wild-type Sox2 caused an activation of the response (Fig. 7B). Similar suppression by Sox2-EnR and activation by wild-type Sox2 were observed with a reporter construct containing the well characterized domain 2* fragment (Fig. 4A) and wild-type Sox2 counteracted the suppression caused by Sox2-EnR (Fig. 7C).

Sox proteins often pair with specific partner factors to regulate target gene transcription (Kamachi et al., 2000). This led to the idea that a mutant construct in which the DNA-binding HMG domain of a Sox protein was deleted could act as a dominant-negative version by competing with the endogenous Sox protein for a specific partner factor (Conlon et al., 1996; Kishi et al., 2000). Indeed, such a dominant-

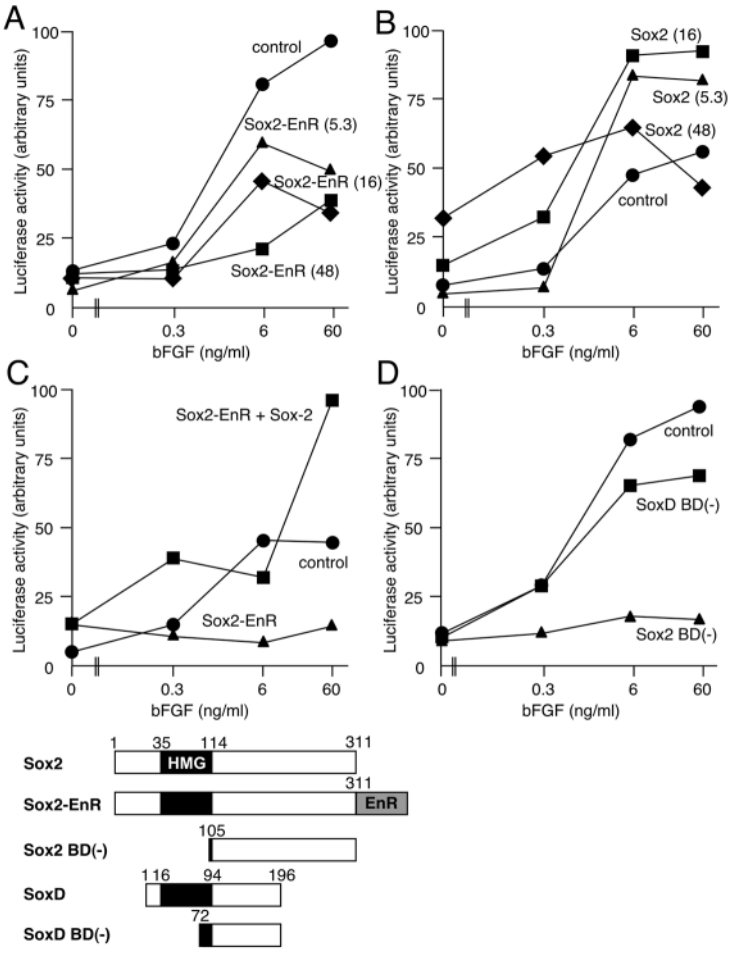

Fig. 7. Involvement of Sox2 as a co-activator in the Fgf response of *Xcad3*. Structural features of Sox2, its derivatives and SoxD BD(–) examined are illustrated at the bottom. DNA-binding HMG domains and EnR repressor domain are marked. Experimental procedures are as described in Fig. 5 except that the reporter construct (–546/LUC/domain2*) was used in C. (A) Effects of dominant negative Sox2 on the Fgf response of *Xcad3/LUC*. The injected amounts were 5.3 (▲), 16 (◆) or 48 (■) pg Sox2-EnR mRNA/blastomere. Sox2-EnR suppresses the Fgf response of the reporter construct (–546/LUC/intron1) in a dose-dependent manner. (B) Effects of wild-type Sox2 on the Fgf response of *Xcad3/LUC*. The injected amounts were 5.3 (▲), 16 (■) or 48 (◆) pg Sox2 mRNA/blastomere. (C) Reversal of Sox2-EnR induced suppression of Fgf response by wild-type Sox2. The injected amounts were 16 pg, 96 pg and 112 pg/blastomere for Sox2-EnR, Sox2 and EnR mRNA. Note that the suppression of reporter gene (–546/LUC/domain2*) expression induced by Sox2-EnR (▲) is reversed by the addition of Sox2 (■). (D) Effects of Sox2 BD(–) (▲) and SoxD BD(–)(■) on the Fgf response of *Xcad3/LUC*. The injected amounts were 160 pg/blastomere for both Sox2 BD(–) and SoxD BD(–) mRNA.

negative version of Sox2 [Sox2 BD(–)] was successfully used to downregulate *Hoxb9* in the posterior neural tube, whereas a similar dominant negative version of SoxD, another Sox family protein that was expressed in the neuroectoderm (Mizuseki et al., 1998b), failed to suppress *Hoxb9* expression (Kishi et al., 2000). In the present embryonic cell culture assays, overexpression of Sox2 BD(–), but not of SoxD BD(–) effectively suppressed the Fgf response of *Xcad3* as expected (Fig. 7D).

Collectively, our functional analysis shows that endogenous Sox2 is required for the Fgf response of *Xcad3*. The most plausible partner factors of Sox2 are Ets proteins, which are also known to require interaction with partner factors to direct signals to specific target genes (Wasylyk et al., 1998). It is highly likely that Sox2 competes with XTcf3 for TLBMs in the composite FREs and cooperate with Ets proteins that bind to adjacent EBMs. To test this idea, an intronic fragment containing one EBM and two TLBMs (overlined in Fig. 4A; probe T2/E4/T3 in Fig. 8A) was examined for its ability to interact with the XTcf3, Sox2 and XEts1 proteins in the gel mobility shift assay. V5-tagged XTcf3 alone shifted the end-labeled probe, yielding three bands (bands 1, 2 and 3 in Fig. 8A, lane 2). All the three bands were supershifted by antibody against V5-epitope (lane 3) and competed by a 125-fold molar excess of the unlabelled probe (lane 4). When the two TLBMs in the probe were mutated (probe E4), no bands emerged (lane 5), but mutation in either TLBM3 (probe T2/E4) or TLBM2 (probe E4/T3) alone gave rise to two bands that co-migrated with bands 1 and 3 (lane 6 and 8), respectively. All these bands were supershifted by the anti-V5 antibody (lane 7 and 9). These results indicated that band 3 was derived from binding of XTcf3 to either TLBM2 or TLBM3, while band 2 was derived from binding of XTcf3 to both motifs. Band 1 was probably formed by binding of multimerized XTcf3 to these motifs. Binding of XTcf3 to TLBMs appeared to be competed by the presence of an increasing amount of Sox2 in the binding reaction (lane 10 to 12). V5-tagged Sox2 alone gave rise to a single band (lane 13), that was abolished by the anti-V5 antibody (lane 14), and did not emerge with the probe E4 (lane 15). We could not detect binding of Ets proteins used in this study with the wild-type probe, but we found that XEts1 was capable of binding to the TLBM3-mutated probe (band 5; lane 2 in Fig. 8B) to which Sox2 also bound (band 6; lane 1). XEts1 and Sox2 proteins appeared to form a ternary complex with this mutated probe (band 4; lane 3), which was supershifted by the anti-V5 antibody (lane 4). These observations strongly support the idea of the direct regulatory function of XTcf3, Sox2 and XEts1 proteins in the Fgf response of *Xcad3*.

We finally asked whether the reporter gene we used (–546/LUC/intron1) was a direct target of Fgf signaling; that is, whether the regulation of the reporter gene by Fgf involved additional intermediates. For this, we cultured ectoderm cells that had been injected with the reporter construct in the presence of bFgf alone or bFgf with the protein synthesis inhibitor cycloheximide (CHX). The concentration of CHX used in this experiment (10 μg/ml) produced more than 90% reduction in translation as previously reported (Isaacs et al., 1998). A high dose of bFgf induced the reporter gene expression in the embryonic cell culture assay within 1.5 hours, even in the presence of CHX (Fig. 9A). The extent of expression obtained in the presence of CHX was comparable to that in the absence of CHX, indicating that the intron 1 in the reporter construct was an immediate early target of Fgf signaling.

Discussion

Integration of multiple signaling pathways on FREs of *Xcad3*

We show that FREs are widely dispersed in intron1 of *Xcad3*. The reporter constructs containing the FREs exhibit high dose dependence on Fgf similar to that shown for endogenous *Xcad3*, when examined in the embryonic cell culture assay. Sequence and mutagenesis analyses reveal that these multiple FREs comprise EBMs and TLBMs that lie in juxtaposition. The EBM is known to serve as the binding site for Ets family transcription

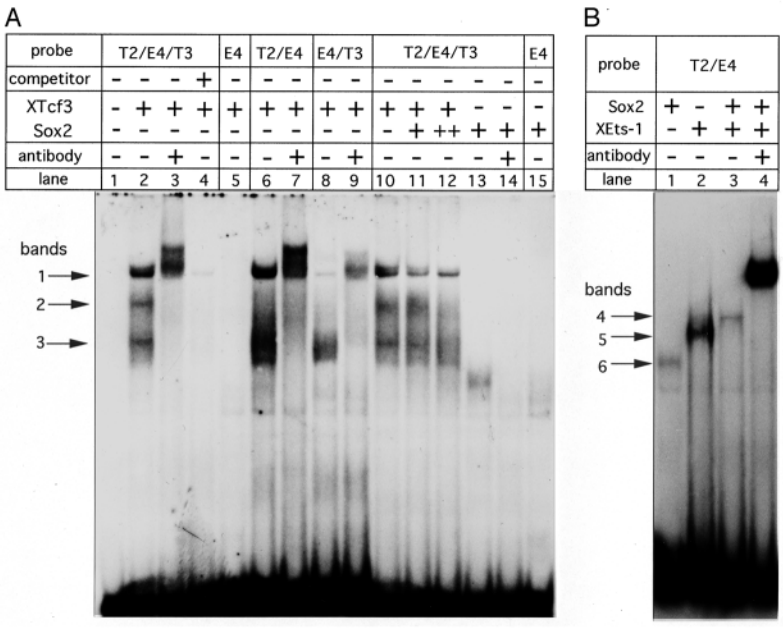

Fig. 8. Direct interaction of EBM and TLBM with XTcf3, Sox2 and XEts1 proteins. An end-labeled probe, either wild type or mutated, was incubated with XTcf3 or Sox2, or with a combination of both proteins in A, and with Sox2 or XEts1, or with a combination of both proteins in B, as indicated in diagrams above. All proteins were tagged with a V5 epitope and made by in vitro translation. Probe T2/E4/T3: an intronic DNA fragment containing TLBM2, 3 and EBM4 (overlined in Fig. 4A). Probe E4: TLBM2 and 3 mutated. Probe T2/E4: TLBM3 mutated. Probe E4/T3: TLBM2 mutated. Mutations were introduced as described in Fig. 4. Unlabeled probe (competitor) or antibody against V5 epitope was added as indicated in the diagrams.

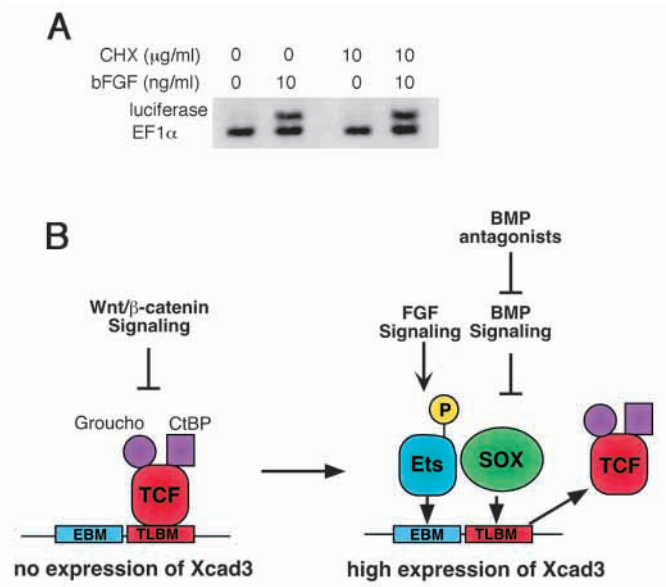

Fig. 9. Intron 1 as an immediate early target of Fgf signaling (A) and a proposed model of integration of multiple signaling pathways on FREs of *Xcad3* intron1 (B). In A, experimental design was essentially the same as in Fig. 2C, except for the following changes. A reporter construct (–546/LUC/intron1) was injected into animal blastomeres of eight-cell stage embryos. Ectodermal cells at stage 10 were cultured for 30 minutes with or without 10 μg/ml cycloheximide and then for an additional 1.5 hours with or without bFgf (10 ng/ml). The transcriptional levels of the reporter gene (luciferase-coding portion) were analyzed by RT-PCR.

factors that are nuclear effectors of the Fgf/Ras/Mapk pathway (Wasylyk et al., 1998). Indeed, functional and physical analyses show that Ets proteins are involved in the Fgf response of *Xcad3* as transcriptional activators, and that *Xcad3* is directly targeted by the Fgf signaling pathway. This conclusion is consistent with the previous observation that Fgf could induce *Xcad3* expression in the animal cap assay within 2 hours of its addition and even in the presence of the protein synthesis inhibitor cycloheximide, which indicates that *Xcad3* is an immediate early target of Fgf signaling (Isaacs et al., 1998).

TLBMs could serve as the binding sites for Tcf/Lef family transcription factors that are nuclear effectors of the Wnt/β-catenin pathway (Molenaar et al., 1996). We had anticipated that XTcf3 functioned as a co-activator of Ets proteins, as Wnt signaling was suggested to be involved in activation of posterior neural genes (Kiecker and Niehrs, 2001; McGrew et al., 1995). Surprisingly, however, functional analysis reveals that XTcf3 acts as a repressor of *Xcad3*. Our data suggest that the endogenous pool of β-catenin in ectoderm cells is considerably smaller compared with that of XTcf3 co-repressors such as XCtBP and Groucho. This in turn implies that Wnt signaling could activate *Xcad3* expression in embryonic cells, when they were provided with larger pool of β-catenin. Marginal zone cells of the early gastrula embryo, where *Xcad3* is initially expressed, are among such candidate cells, as a relatively large amount of β-catenin is translocated into the nucleus in these cells (Schohl and Fagotto, 2002). Recently, an mutant function of Tcf3 as a repressor is revealed in the zebrafish *headless* mutant that carries a mutation in *Tcf3* (Kim et al., 2000). In this mutant, expression of midbrain-hindbrain boundary genes such as En2 and Pax2 are de-repressed in more anterior neural region, leading to severe head defects. It would be interesting to know whether similar anterior expansion is seen in Cdx gene expression in this mutant.

Sox2 is de-repressed by Bmp antagonists in the neurogenic region of ectoderm during neural induction (Mizuseki et al., 1998a). We show that Sox2 which shares a cognate DNA bindings motif with Tcf/Lef family members, is required as a co-activator for the Fgf response of *Xcad3*. Sox2 is likely to compete with XTcf3 for TLBMs in the composite FREs to cooperate with Ets proteins that bind to adjacent EBMs. Physical analysis supports this idea. Both Sox and Ets family transcription factors interact with specific partner factors to direct signals to target genes (Kamachi et al., 2000; Wasylyk et al., 1998), but direct partnership between them has not been reported. Collectively, our results indicate that signaling pathways of Fgf, Bmp and Wnt are integrated on the FREs to regulate the expression of *Xcad3* in the posterior neural tube through positively acting Ets and Sox proteins and negatively acting Tcf protein (Fig. 9B).

Fgf as a morphogen

Ets (Chen et al., 1999) and Sox (Mizuseki et al., 1998a; Mizuseki et al., 1998b) proteins are ubiquitously expressed in the neurogenic region during gastrula stages when neural patterning is initiated. Posteriorly biased *Xcad3* expression could, therefore, be primarily due to similarly biased expression of Fgf proteins. Indeed, several Fgf genes are activated in the posterior ectoderm and mesoderm during late gastrula and early neural stages (Christen and Slack, 1997; Isaacs et al., 1992; Tannahill et al., 1992). In this and previous studies (Kengaku and Okamoto, 1995), we have shown that Fgf can induce gastrula ectoderm cells to express position-specific neural marker genes along the AP axis in a dose-dependent manner, with higher doses eliciting more posterior neural genes. Interestingly, functional analysis indicated that Sox-mediated signaling (Kishi et al., 2000; Mizuseki et al., 1998b) and Fgf signaling (Hongo et al., 1999) were also required for the expression of anterior neural genes. These studies raise the possibility that regulatory mechanisms underlying the transcriptional activation of anterior neural genes possess common features with that for *Xcad3* activation except for higher sensitivity to Fgf. Differential sensitivity of position-specific neural genes to Fgf would then imply that Fgf acts as a morphogen during neural patterning (Kengaku and Okamoto, 1995). To explore this issue, we need to identify *cis*-elements in anterior neural genes, and the present embryonic cell culture assay system will be useful for this purpose.

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