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RAPID COMMUNICATION

A Novel TGF-β-like Gene, *fugacin*, Specifically Expressed in the Spemann Organizer of *Xenopus*

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Using a differential screening strategy, we have cloned a novel *Xenopus* gene, *fugacin*, related to the transforming growth factor *β* superfamily. Transcripts were detected primarily in the dorsal marginal zone of late blastula. Thereafter, they became highly localized to the blastopore lip of early gastrula and were not observed at later stages. This gene, which is most homologous to the mouse gene *nodal*, displays a new pattern of cysteine residues. These findings highlight the potential role of these growth factors during early vertebrate development.

INTRODUCTION

The molecular basis of the spectacular organizing activity demonstrated long ago in transplantation experiments on amphibian embryos (Spemann and Mangold, 1924) remains incompletely understood. However, the recent discovery of several potential patterning molecules has begun to yield insights into this process. In *Xenopus laevis*, several genes transcribed predominantly within the gastrula organizing region have been isolated. These genes encode either putative transcription factors, such as *goosecoid*, *Xlim-1*, *XFKH-1*, *Pintallavis*, and *Xnot*, or secreted proteins such as noggin and chordin (recently reviewed by Dawid, 1994; Kessler and Melton, 1994). However, overlapping and functionally redundant signaling systems clearly operate at many steps of embryonic pattern formation. To decipher such functional networks of gene interactions and regulation will clearly require the identification and characterization of many involved factors.

Here, we present the molecular cloning and sequence analysis of a novel transforming growth factor *β* (TGF-*β*)-like gene. Expression of this gene, which we have named *fugacin*, is spatially and temporally localized to the organizer region.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. U295880.

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MATERIAL AND METHODS

Embryo Manipulations

Embryos were staged according to Nieuwkoop and Faber (1967).

UV-ventralized embryos were obtained by irradiating their vegetal poles for 5 min in a quartz chamber using UVG11 (UVP Inc., San Gabriel, CA), 20–25 min after fertilization.

Dorsalized embryos were obtained by 0.3 *M* LiCl treatment in 10% NAM for 6 min at the 32-cell stage.

Isolation of Dorso-Specific cDNAs

cDNAs from a blastopore lip Lambda ZAP library (Blumberg *et al.*, 1991) were excised as pBluescript SK subclones according to Stratagene, using R408 helper phage and the XL1Blue bacterial strain. Colonies were plated onto nitrocellulose filters, and then duplicated filters were hybridized

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with PCR probes enriched for dorsal (D probe)- or ventral (V probe)-specific cDNAs (see below for the preparation).

Colonies that gave a strong signal only with D probes were screened again with known dorsal-specific sequences. Clones that did not shown hybridization were partially sequenced and compared to the data banks.

Preparation of the Probes Enriched with Dorsal- and Ventral-Specific cDNAs

Total RNA was isolated from individual explants extirpated from the dorsal (D) or ventral (V) marginal zones at the early gastrula stage by a previously described method (Zaraisky *et al.*, 1992). The first-strand cDNA synthesis was performed according to a standard protocol (Amersham) in the presence of 30 pmole of T-primer (CGCAGTCGACCG(T)13).

The cDNA first strands were precipitated twice with ethanol and oligo(dA)-tailed with dATP according to a standard protocol (Promega). PCR of the tailed D and V samples was performed with T-primer exactly as described (Lukyanov *et al.*, 1995).

Reciprocal subtractive hybridization of D and V samples was done according to the earlier published protocol (Lukyanov *et al.*, 1994). As a result, two samples of cDNA enriched in dorsal or ventral sequences were obtained.

Whole-Mount in Situ Hybridization

In situ hybridization was performed essentially as described by Harland (1991), except that BM purple was used (Boehringer) for alkaline phosphatase substrate.

Digoxigenin-labeled RNAs were prepared by *in vitro* transcription in the presence of digoxigenin–UTP (Boehringer), using T7 and T3 RNA polymerases, respectively, for antisense and sense probes.

cDNA Sequencing

Selected cDNAs were sequenced on both strands by the dideoxy chain termination method (Sanger *et al.*, 1977) using a Sequenase sequencing kit (from Stratagene).

RESULTS AND DISCUSSION

In an attempt to isolate new factors potentially involved in conferring organizer activity, we performed a differential screen for zygotic dorsal-specific cDNAs of a dorsal lip cDNA library derived from stage 10.25 *Xenopus* gastrula (Blumberg *et al.*, 1991). Probes were prepared from one explant of either the dorsal or the ventral marginal zone of a stage 10.25 gastrula by a PCR-based method. In order to increase the screening efficiency, the probes were enriched in either dorsal- or ventral-specific cDNAs by subtractive hybridization as described under Material and Methods. After an initial differential screening of 10,000 colonies, 15 clones showed a clear preferential hybridization signal with a dorsally derived probe. To identify the cDNAs of interest, their reactivity to known dorso-specific sequences was checked. This led us to characterize three groups: five clones hybridized with the organizer-specific homeobox gene *goosecoid*, and nine others correspond to the recently identified novel factor chordin (Sasai *et al.*, 1994). The third group is represented by a single cDNA. This clone did not contain sequences corresponding to a known gene. We named this novel gene *fugacin*, taking its expression pattern into account.

In order to obtain a greater number of cDNAs, a further screening of the dorsal lip library was performed with a *fugacin*-derived probe. From 150,000 plaques screened, 8 positive clones were selected. The longest fugacin insert, 1.6 kb, was sequenced. The longest open reading frame encodes a protein of 400 amino acids (Fig. 1A), displaying the hallmarks of the TGF-β superfamily (Fig. 1B). The predicted protein contains a NH2-terminal hydrophobic region suggestive of a signal sequence for secretion and two putative tetrabasic processing sites (RRLRR) at residues 272 or 273. Cleavage of the precursor at these sites would be expected to generate a mature domain 128 or 127 amino acids in length. Alignment of the COOH-terminal Fugacin sequence with the corresponding region of several members of the TGF-β superfamily (Fig. 1C) shows significant homologies. Nevertheless, Fugacin displays an altered pattern of cysteine residues. First, the seventh of the seven conserved cysteines is replaced in Fugacin by a phenylalanine. Second, the fourth cysteine is two amino acids upstream of the usual position. Such a cysteine pattern renders Fugacin unique compared to the other known members of the TGF-β family, although some variations in these positions have been recently reported. In particular, the two TGF-β-related proteins Vgr2/GDF3 and GDF9 (Jones *et al.*, 1992; McPherron and Lee, 1993) lack the fourth cysteine involved in the sole disulfide linkage between subunits in other members (Schlunegger and Grutter, 1992). Further studies are needed to understand the functional significance of such variations in the cysteine pattern.

The *in vivo* expression pattern was analyzed by whole-mount *in situ* hybridization. Transcripts were detected at stage 9, following the midblastula transition (Figs. 2A and $2A_1$), in a region limited to the presumptive dorsal part of the embryo at the level of the equatorial zone (dorsal marginal zone). At the onset of gastrulation (stage 10.25), the hybridization appeared to be highly localized as a narrow ring of staining at the top of the dorsal blastopore lip (Figs. 2B and $2B_1$). Some faint punctate staining can be observed in the dorsal endodermal region of embryos. As gastrulation progresses, the signal decreases abruptly: subsequently, no staining was detected either in the horseshoe-shaped blastopore-stage embryos or in later stages (neurula and tailbud). To refine the localization of stained cells, stage 10.25 embryos were sectioned after whole-mount *in situ* hybridization. Cells composing the external layer at the top of the forming blastopore lip are stained, while no staining was observed in deeper cell layers (Fig. 2E).

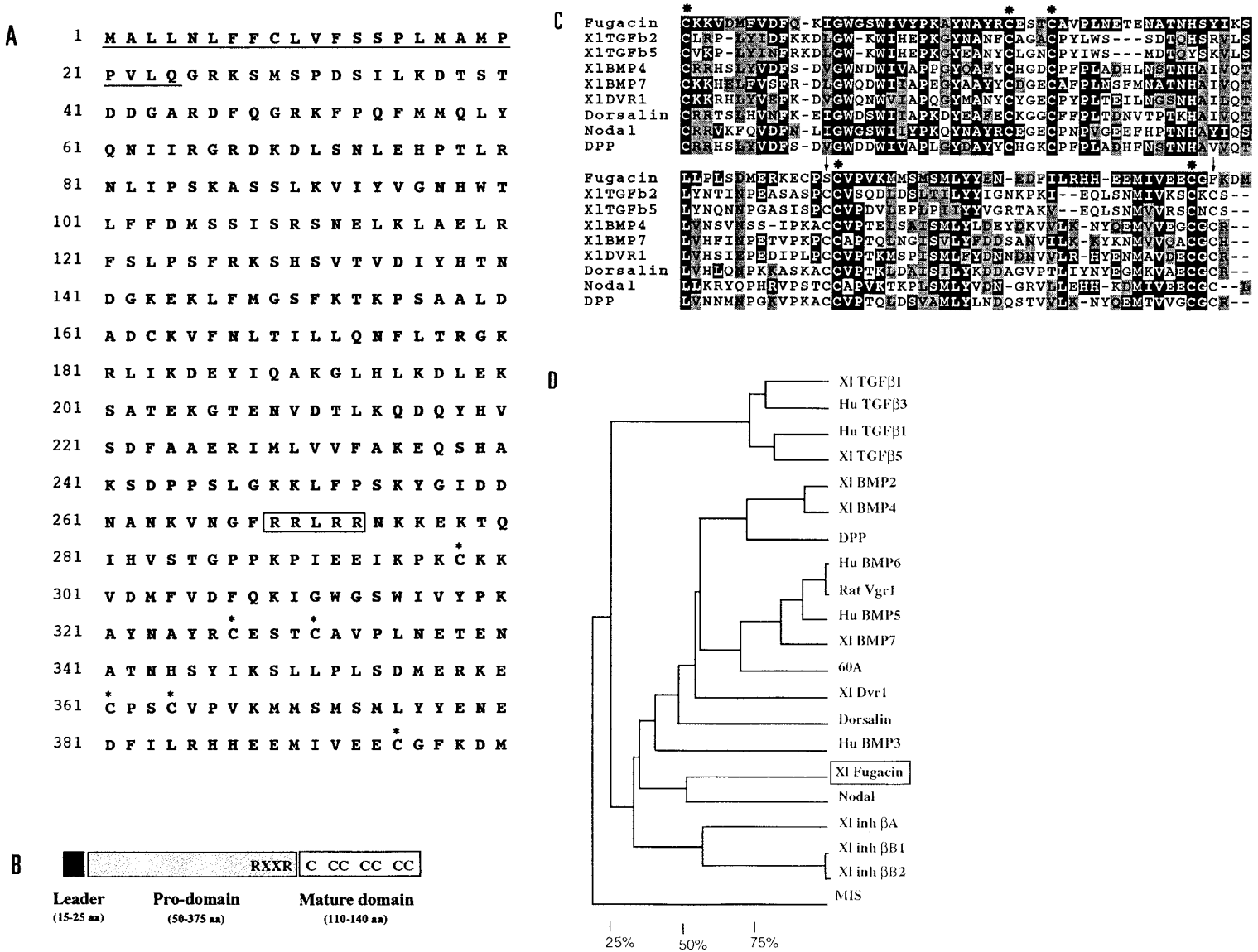

FIG. 1. Sequence of Fugacin and comparison with the TGF-β superfamily. (A) The deduced amino acid sequence of the longest *fugacin* cDNA clone is shown. A putative signal sequence is underlined and the potential cleavage site is boxed. Cysteines within the mature domain are marked by asterisks. (B) Structural representation of the TGF-β superfamily members (Kingsley, 1994). (C) Alignment of the seven-cysteine domain in the TGF-β family. Conserved amino acids are printed on a black background. Residues homologous to those of Fugacin are printed on a gray background. The five conserved cysteines are marked with asterisks. Gaps introduced to optimize the alignment are represented by dashes. Cysteines from Fugacin that differ from the family are indicated by arrows. (D) Graphical representation of the sequence relationships between members of the TGF-β superfamily. The data used for this dendogram have been generated with the Clustal V program. For both the tree and the amino acid homologies, only sequence of the C-terminal domain was used, starting with the first cysteine from the active domain (see text).

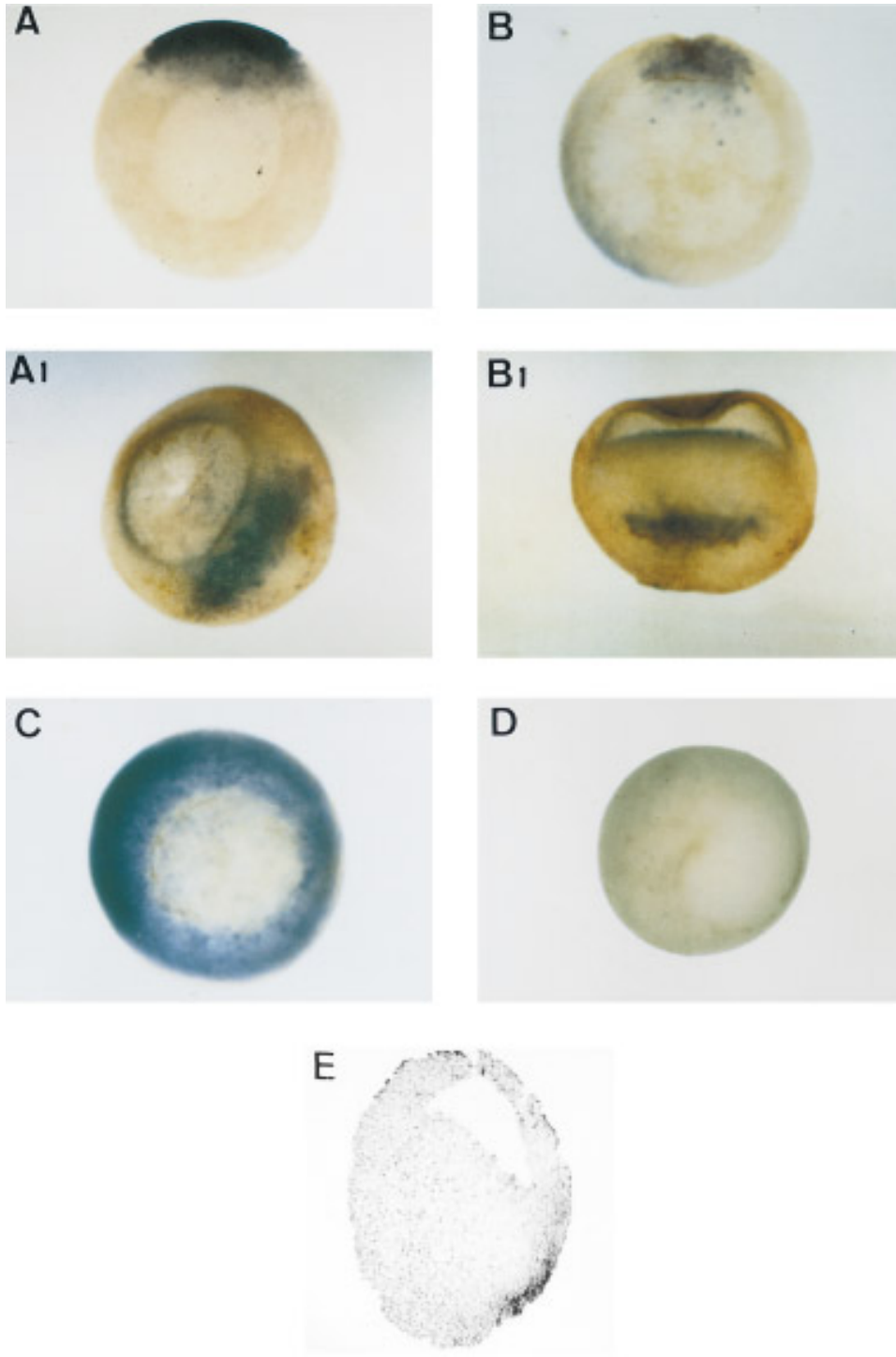

FIG. 2. Whole-mount *in situ* hybridization with a *fugacin* antisense riboprobe labeled with digoxigenin. RNA is transiently expressed in the Spemann organizer. (A) Albino embryo at late blastula (stage 9), vegetal view; dorsal is up. (A1) Dorsal view. Staining is present in the dorsal mesoderm. (B) At early gastrula (stage 10.25), vegetal view; dorsal is up. (B1) Dorsal view. Staining is restricted to the top of the blastopore lip. Note that a few vegetal cells are also labeled. (C) Dorsalized LiCl-treated embryo, vegetal view. Staining appears throughout the marginal zone. (D) Ventralized UV-treated embryo, vegetal view. No staining is visible. (E) Transverse histological section after whole-mount *in situ* hybridization at stage 10.25. Staining is restricted to the Spemann organizer.

We then asked whether the localized expression of *fugacin* mRNA was dependent on a dorsal identity of expressing cells. Several experimental treatments are known to affect the dorsoventral polarity of the embryos (LiCl treatment and UV irradiation), and these have a marked effect on the expression pattern of our gene as revealed by *in situ* hybridization. Figures 2C and 2D show vegetal pole views of LiCl- or UV-treated embryos, respectively. In dorsalized LiCl-treated embryos, the staining is intense throughout the marginal zone. Inversely, UV treatment that induces ventralization is sufficient to totally abolish *fugacin* gene expression. These data appear to confirm the dorsal-specific expression of *fugacin*.

The expression pattern of *fugacin* is different from that of the TGF-β-related factors which were previously identified during *Xenopus* development. A key observation reported here for *fugacin* mRNA is its highly restricted expression in both time and space. This pattern is very reminiscent of that of the mouse *nodal* gene (Zhou *et al.*, 1993). In this respect, it is interesting to note that the calculated phylogenetic relationships in the C-terminal regions between the members of the TGF-β superfamily (Fig. 1D) indicate that Fugacin has the strongest sequence identity with Nodal (53%). All these findings suggest that in amphibians an additional TGF-β-related factor could play a role in the organizing capacity of Spemann's organizer. Thus, it seems increasingly likely that members of this family of growth factors are involved in many embryological events which control the formation of body axes in vertebrates.

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Note added in proof. While the manuscript was in press, a paper about the same gene, Xnr3, was published by the R. Harland group.

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