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Systematic Screening and Expression Analysis of the Head Organizer Genes in *Xenopus* Embryos

Mikihito Shibata,*,† Mari Itoh,*,† Shin-ya Ohmori,*,† Jun Shinga,*,† and Masanori Taira*,†,[1]

**Department of Biological Sciences, Graduate School of Science, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan; and †CREST, Japan Science and Technology Corporation, Japan*

We describe here a systematic screen of an anterior endomesoderm (AEM) cDNA library to isolate novel genes which are expressed in the head organizer region. After removing clones which hybridized to labeled cDNA probes synthesized with total RNA from a trunk region of tailbud embryos, the 5′ ends of 1039 randomly picked cDNA clones were sequenced to make expressed sequence tags (ESTs), which formed 754 tentative unique clusters. Those clusters were compared against public databases and classified according to similarities found to other genes and gene products. Of them, 151 clusters were identified as known *Xenopus* genes, including eight organizer-specific ones (5.3%). Gene expression pattern screening was performed for 198 unique clones, which were selected because they either have no known function or are predicted to be developmental regulators in other species. The screen revealed nine possible organizer-specific clones (4.5%), four of which appeared to be expressed in the head organizer region. Detailed expression analysis from gastrula to neurula stages showed that these four genes named *crescent*, P7E4 (homologous to human hypothetical genes), P8F7 (an unclassified gene), and P17F11 (homologous to human and *Arabidopsis* hypothetical genes) demarcate spatiotemporally distinct subregions of the AEM corresponding to the head organizer region. These results indicate that our screening strategy is effective in isolating novel region-specific genes.

***Key Words:* *Xenopus laevis*; Spemann organizer; head organizer; anterior endomesoderm; regional ESTs; expression pattern screen; head organizer-specific genes.**

INTRODUCTION

Spemann and Mangold discovered the inductive properties of the organizer in amphibian embryos by implanting the dorsal blastopore lip of a donor embryo on the ventral side of a second embryo (Spemann and Mangold, 1924). O. Mangold further established the notion that the organizer can be subdivided into the head organizer, which induces the prospective anterior brain structure, and the trunk organizer, which induces the prospective hindbrain and spinal cord. Thus, the organizer plays important roles not only in tissue induction but also in establishing the anteroposterior axis (Mangold, 1933). In the last decade, several genes expressed in the organizer have been isolated, and their functions have been studied. Those genes encode secreted molecules, mostly BMP or Wnt inhibitors, and transcription factors, mostly homeodomain proteins such as Goosecoid (Cho *et al.*, 1991), Xlim-1 (Taira *et al.*, 1992), Xanf-1 (Zaraisky *et al.*, 1992), Otx-2 (Blitz and Cho, 1995; Pannese *et al.*, 1995), and XHex (Newman *et al.*, 1997). According to the two-inhibitor model, head formation is brought about by repression of both BMP and Wnt signals by their secreted antagonists (Glinka *et al.*, 1997). Known Wnt antagonists include Cerberus (Bouwmeester *et al.*, 1996), Dkk-1 (Glinka *et al.*, 1998), and Frzb-1 (Leyns *et al.*, 1997; Wang *et al.*, 1997a), which are expressed mainly in the head organizer region.

In addition to the prechordal plate, which has been thought to be the head organizer, the importance of the anterior endodermal region in head induction has been postulated based on studies of the head inducer Cerberus, which is expressed in the anterior endoderm of *Xenopus* embryos (Bouwmeester *et al.*, 1996; Piccolo *et al.*, 1999). Cerberus has been shown to initiate head formation

[1] To whom correspondence should be addressed. Fax: +81-3-5841-4434. e-mail: m_taira@biol.s.u-tokyo.ac.jp.

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when expressed in the ventral region (Bouwmeester *et al.*, 1996) and to interact with BMP, Wnt, and Nodal to inhibit their signaling (Piccolo *et al.*, 1999). In addition, studies in mouse have indicated that the anterior visceral endoderm (AVE) may play a role in head formation (Thomas and Beddington, 1996), for ablation of the AVE interferes with proper head formation. Although these studies present a framework for signals involved in head induction, the gene cascades required for the formation of the head organizer and the induction of brain structures are not fully understood. First, how are the anterior endoderm, prechordal plate, and notochord specified by transcription factors and secreted molecules? So far, some models have been proposed for the interactions or relationships of organizer-specific transcription factors in the specification of dorsoventral and anteroposterior mesoderm (Artinger *et al.*, 1997; Mochizuki *et al.*, 2000; Taira *et al.*, 1997). As for specification of the anterior endomesoderm, the roles of transcription repressors such as Goosecoid (Artinger *et al.*, 1997) and XHex (Brickman *et al.*, 2000) have been described. Second, how is the anterior neural plate regionalized by factors that emanate from the head organizer? The initial acquisition of regionalization of anterior neural plate may not be explained simply by the inhibition of BMP and Wnt signaling. The existence of positive factors that induce the regionalization of neural plate is conceivable.

To explore further the molecular and functional complexity of the organizer, it would be valuable to isolate as many novel markers or developmental regulators as possible that are specific to the organizer and to elucidate the relationships between different organizer genes. Expression pattern analysis appears to be suited for a systematic screen, since several genes which play important roles in development have been isolated by this approach (Gawantka *et al.*, 1998). However, a tremendous number (several thousands to several ten thousands) of clones may need to be analyzed to isolate genes of interests. We therefore conducted a relatively small-scale screen (approximately 1000 clones) using a region-specific cDNA library as a gene source for expression pattern screening. To isolate head organizer genes, we constructed an anterior endomesoderm (AEM) cDNA library and carried out expression pattern screening by whole-mount *in situ* hybridization. To increase the efficiency of screening, we also created expressed sequence tags (ESTs) based on partial sequence of each picked clone from the AEM cDNA library before expression pattern screening. EST data allowed us to reduce redundant clones and to eliminate housekeeping genes or known *Xenopus* genes without loosing rare ones. Here, we present the screening strategy in detail and the isolation of four genes, including a previously reported gene, *crescent* (Shibata *et al.*, 2000), that show specific expression patterns in the AEM which corresponds to the head organizer region.

MATERIALS AND METHODS

Manipulation of Xenopus Embryos

Unfertilized eggs were squeezed out manually from pigmented or albino female *Xenopus laevis* which had been injected with gonadotropin (Teikokuzoki Co.). Eggs were artificially fertilized with testis homogenates, dejellied in 2% L-cysteine-HCl (pH 7.9), and reared in 0.1× Steinberg's solution at 14–21°C until the desired stages. Embryos were staged according to Nieuwkoop and Faber (1967). To isolate the AEM and anterior neuroectoderm (ANE) regions, anterodorsal parts of embryos were dissected with microscissors and forceps as illustrated in Fig. 1, treated with 0.5 mg/ml of Collagenase Type I (Sigma) in 1× MBS (modified Barth's saline), and manually separated into AEM and ANE portions.

Construction of Unidirectional cDNA Library

Total RNA was extracted from the collected AEM region by guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987), and poly(A)$^+$RNA was further isolated by using a column loaded with Oligo(dT) cellulose (Sigma) with 0.8–1.0% recovery. A unidirectional AEM cDNA library was constructed with 0.8 μg of poly(A)$^+$ RNA by using a ZAP cDNA synthesis kit (Stratagene). The insert sizes of the constructed cDNA library were 1.5 kb on average and the library contained 600,000 plaque forming units.

Plaque Hybridization

Plaques were transferred onto nitrocellulose membranes (Schleichler and Schuell), and blots were hybridized with ^{32}P-labeled cDNA probe generated with 50 μg of total RNA from the trunk and tail regions posterior to the otic vesicles of stage-26 embryos.

PCR and Purification of the Product

cDNA clones were amplified by PCR using SK (5′-TCTAGAACTAGTGGATC-3′) and T7 (5′-CCGGATCCTAATACGACTCACTATA-3′) primers in 96-well plates. PCR products were purified by a method using saturated KI solution and 96-well FB plates (Millipore) according to the manufacturer's protocol, and residual KI was removed with HV plates (Millipore) loaded with Sephadex G-50 (Pharmacia).

DNA Sequence Analysis

Purified PCR products were sequenced from the 5′ end with a semiautomated sequencer (ABI310, Perkin Elmer) using the SK primer. The average size of informative data was 550 nucleotides. For further analysis, plasmid excision from lambda-ZAP clones was carried out by using an ExAssit/SOLR system (Stratagene) in the form of pBluescript SK(−). The entire DNA sequence of inserts was determined by a LiCor 4200 sequencer (Aloka) using T3 primer (5′-AATTAACCCTCACTAAAG-3′), T7 primer (TAATACGACTCACTATAGGG), and their deleted plasmid constructs as templates. Sequences were clustered and compared to public DNA or protein sequence databases. ESTs with an E value of less than e-15 as determined by the blast search were classified as "similar" (see Results) and automatically compiled in a local database using the

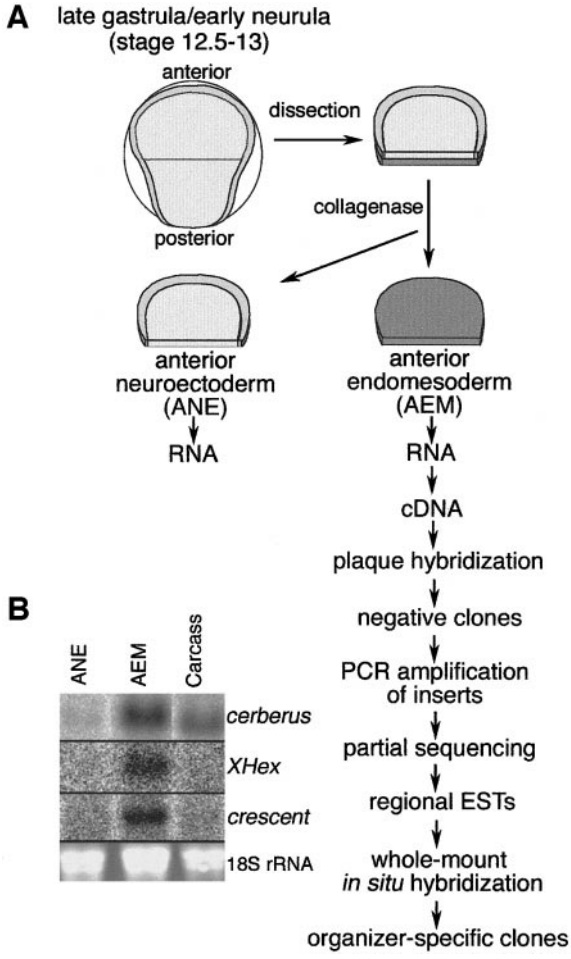


FIG. 1. Schematic representation of screen strategy. (A) Isolation of AEM by dissection and screen strategy. Late gastrulae/early neurulae (stage 12.5–13) were first cut half vertically along the anteroposterior axis. Dorsal–anterior parts were obtained by cutting as illustrated. These pieces contain anterior neuroectoderm (ANE), anterior endomesoderm (AEM), and epithelia covering the archenteron roof. Dissected pieces were treated with collagenase and separated into ANE and the underlying AEM. A region-specific cDNA library was constructed from the AEM RNA. Further detailed processes are described in the text. (B) The efficiency of dissection. Northern blot analysis was performed by using 10 μg of total RNA isolated from ANE, AEM, and carcass. A blot was hybridized with probes of organizer-specific genes, *cerberus, XHex*, and *crescent*, sequentially. Probes used are indicated on the right side of the panel. Ethidium bromide-stained 18S rRNA was used as loading control.

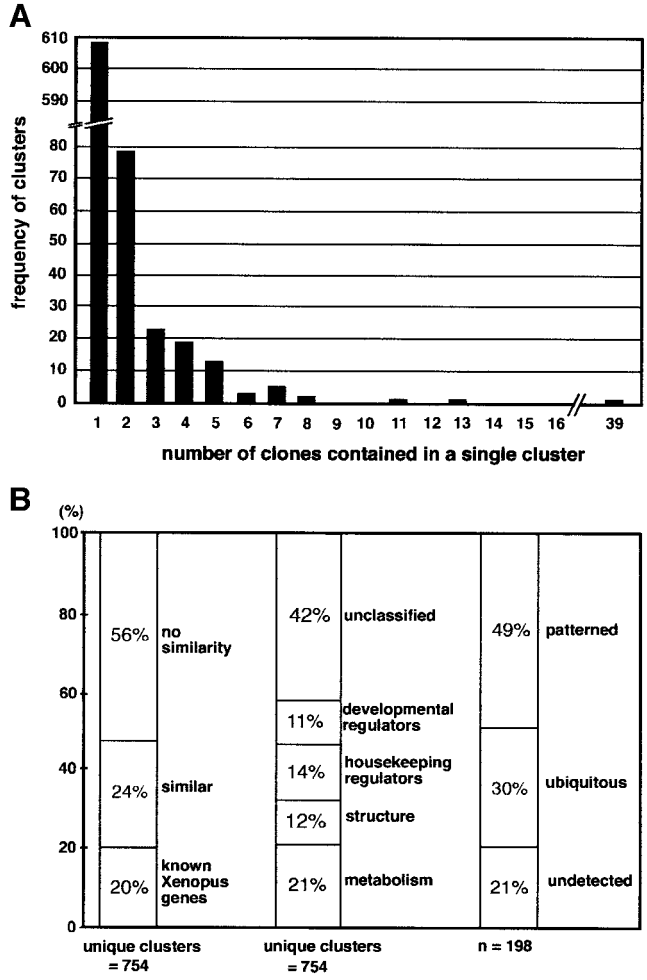


FIG. 2. Clustering and classification of isolated clones. (A) Unique clusters (754) were classified according to the number of clones contained in a single cluster. Ordinate, the frequency of cluster; abscissa, number of clones contained in a single cluster. (B) Classification of sequence data and expression pattern of clones isolated from an AEM cDNA library. Unique clusters (754) were classified according to the similarity found to nucleotide sequences of *Xenopus* or other species genes (left column), or predicted/established functions of translated products (middle column) on the basis of the results of a homology search against DNA or protein databases, respectively. Clones were structurally divided into three categories: "no similarity," "similar," and "known *Xenopus* genes," and functionally divided into "unclassified" (functionally unknown), "developmental regulators" (predicted or established functions as regulators in development), "housekeeping regulators" (predicted to be housekeeping regulators necessary for cell survival, such as splicing factors, elongation factors, cell division proteins, and so on), "structure" (involved in cell motility, cytoskeletal or extracellular matrix components or histones), "metabolism" (housekeeping enzymes or transporters). Out of 354 functionally unclassified or possible developmental regulators, 198 were analyzed for expression patterns by whole-mount *in situ* hybridization, and classified into three categories: "patterned," "ubiquitous," and "undetectable" (right column). Values are given as percentages of total number of clones examined in each column.

Xenopus database system XEXTDB, which was converted from the *Caenorhabditis elegans* database system NEXTDB (constructed by Y. Kohara and T. Shin-i, National Institute of Genetics).

Whole-Mount in Situ Hybridization

Purified PCR products, which possess a T7 RNA polymerase site at the 3′ end (see above), were used to synthesize antisense

TABLE 1

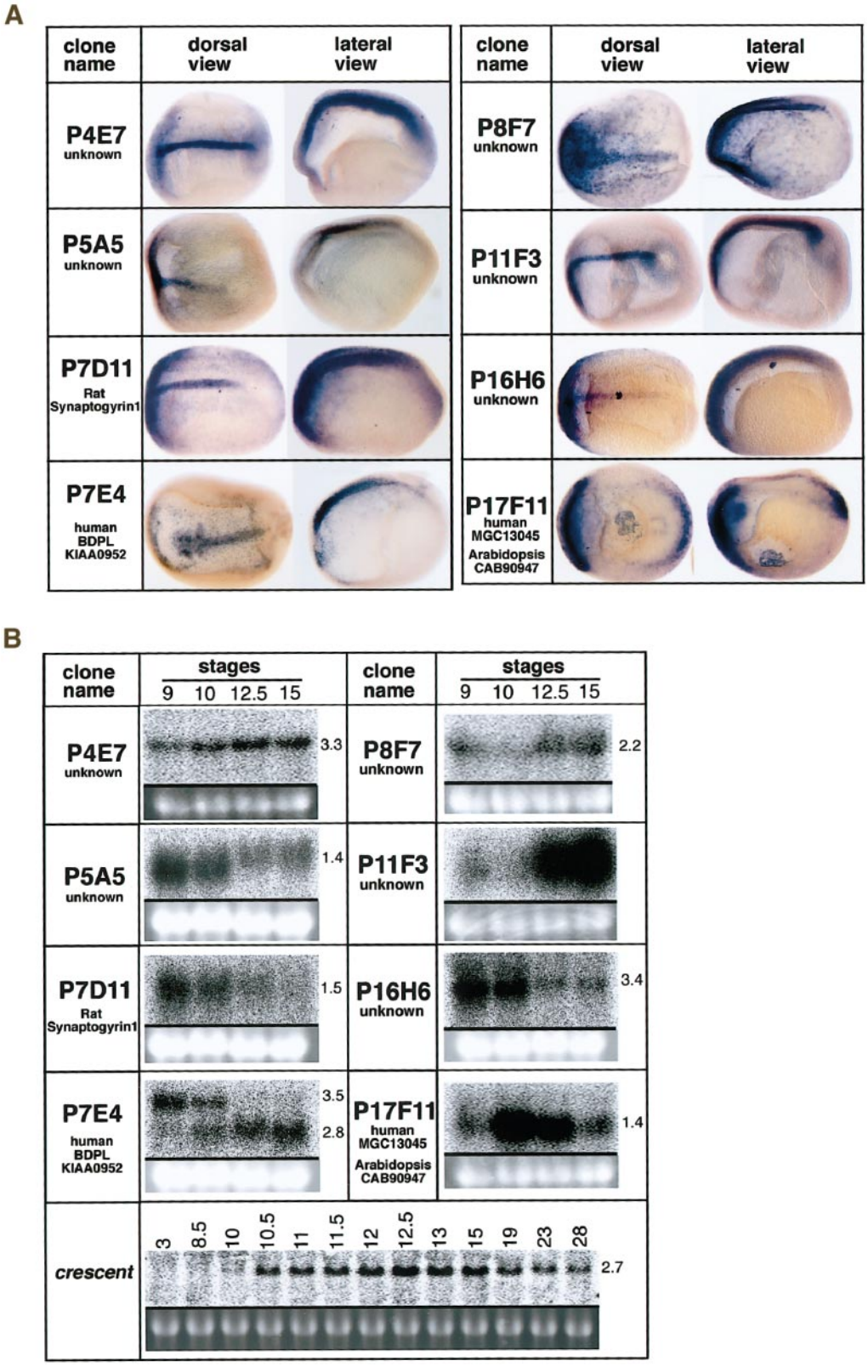
Summary of Isolated Known *Xenopus* cDNAs Which Were Reported to Show Regionalized Expression Patterns

Genes	Gene products	Characterization	No. of times isolated	Localization		
				org	np	other
Xbra	T-box protein	transcriptional activator	1	O		
XGATA2	zinc finger protein	transcriptional activator	1	O		O
Xsox17-alpha	HMG domain protein	transcriptional activator	4	O		O
XHEX	homeodomain protein	transcriptional repressor	1	O		
RAR γ-2	nuclear receptor	transcription factor	1	O		
chordin	secreted protein	BMP inhibitor	3	O		
noggin	secreted protein	BMP inhibitor	1	O		
follistatin	secreted protein	Activin/BMP inhibitor	1	O		
xFRP	secreted protein	Activin/BMP inhibitor?	1	O		
XRhoA	small GTPase	signal-transducing protein	1	O	O	
xFKBP	FK 506-binding protein	signal-transducing protein?	2	O		
XCYP-26	RA-metabolizing enzyme	inhibition of RA signal	1	O	O	
endodermin	secreted protein	protease inhibitor	1	O		O
XK endo B	keratin type I	structural protein	9	O		
PV.1	homeodomain protein	transcriptional repressor	1			O
Gli4	zinc finger protein	transcriptional repressor	1			O
iroquois3	homeodomain protein	transcription factor	1		O	O
HOX-B9	homeodomain protein	transcription factor	1		O	O
Sox D	HMG domain protein	transcription factor	2		O	
XFD-11	winged-helix protein	transcription factor	1			O
XFD-13	winged-helix protein	transcription factor	1			O
MyoD	bHLH protein	transcription factor	1			O
Notch	receptor	signal-transducing protein	1		O	
Xwnt-8B	secreted protein	signal-transducing protein	1		O	O
FrzA	secreted protein	Wnt inhibitor	1			O

Note. Xenopus genes reported to show regionalized expression patterns are listed and classified into organizer (org), neural plate (np), and other region (other) according to their expression domains. O, expression reported around late gastrula and early neurula stages; blank, expression not reported around late gastrula and early neurula stages; org, organizer region; np, neural plate; other, other regions.

DIG-RNA probes by using an *in vitro* RNA synthesis kit (Boehringer Mannheim) with T7 polymerase. Embryos were fixed with MEMFA for the *in situ* hybridization. For hybridization with each probe, two albino embryos of each gastrula/neurula (stages 12–13) and mid-neurula (stages 15–16), and one albino embryo of each late neurula (stages 20–21) and tailbud embryo (stages 25) were used. Early gastrula embryos (stages 10–10.5) were used when necessary. For hemisections, rehydrated embryos were cut with a razor blade in 1× PBS, 0.1% Tween 20, or embedded in 2% low melting point agarose (GIBCO/BRL), 1× PBS, 0.2 M sucrose, 0.1% SDS, then cut with a razor blade. Whole-mount *in situ* hybridization was performed manually or with an automated ISH system (AIH-101, Aloka) according to the method of Harland (1991). Stained embryos were treated with benzyl benzoate/benzyl alcohol (BB/BA) to become transparent. Stained whole or hemisectioned embryos in methanol or ones cleared with BB/BA solution were examined with a Stemi SV11 stereo microscope (Carl Zeiss), and sections of stained embryos were examined with an Axioplan 2 microscope (Carl Zeiss). Images were taken with a digital camera, AxioCam (Carl Zeiss) or ProgRes 3008 (Carl Zeiss), and processed on Adobe Photoshop. In some cases, to orient dehydrated whole or hemisectioned embryos for taking photos, samples were placed in small holes on a 1% agarose plate; suitable-sized holes were made on an agarose plate with a heated glass rod, then the holed agarose plate

FIG. 3. Isolated cDNA clones expressed in the AEM region and notochord. (A) Clone names and similar genes or proteins in the public databases are indicated on the left side of each panel. Results of whole-mount *in situ* hybridization for early neurula (stage 13) are shown in dorsal (left) and lateral (right) view. (B) Northern blot analysis. Clone names and their closest genes or proteins in the public databases are indicated on the left side of each panel and the deduced mRNA size (kb) of each clone on the right (see Table 2 for P11F3). The developmental stages are indicated on the top of each column by numerical figures according to Nieuwkoop and Faber (1967). 18S rRNA stained with ethidium bromide is for loading control. Ten micrograms of total RNA from stage-9, -10, -12.5, and -15 embryos were electrophoresed in each lane, blotted, and hybridized with probes derived from each clone except for *crescent*. *Crescent* probe was hybridized to the same blot as previously used (Hikasa and Taira, 2001) with a wider range of stages as indicated.



was soaked with methanol several times for dehydration before use. A *XHex* cDNA clone (1.4-kb insert) was obtained in this study and used as a probe for whole-mount *in situ* hybridization as well as Northern hybridization.

Sectioning

Stained embryos were embedded in paraffin and sectioned at 15 μm. Nuclei were stained with 400 ng/ml DAPI (Sigma) in 2× SSC.

Northern Hybridization

Procedures of Northern blot hybridization were as described previously (Taira *et al.*, 1994a,b). Hybridized blots were exposed to BAS-IP MN 2041 (Fuji Film) and analyzed with BAS-2500 (Fuji Film).

Quality Control

To confirm that PCR products and DIG-RNA probes used for expression pattern screening were derived from the identical clone, they were electrophoresed on agarose gels and the length of inserts and probes were compared. No inconsistencies were found. In addition, for the organizer-specific clones, it was confirmed that the same expression patterns reproducibly appeared in at least two trials of whole-mount *in situ* hybridization.

RESULTS

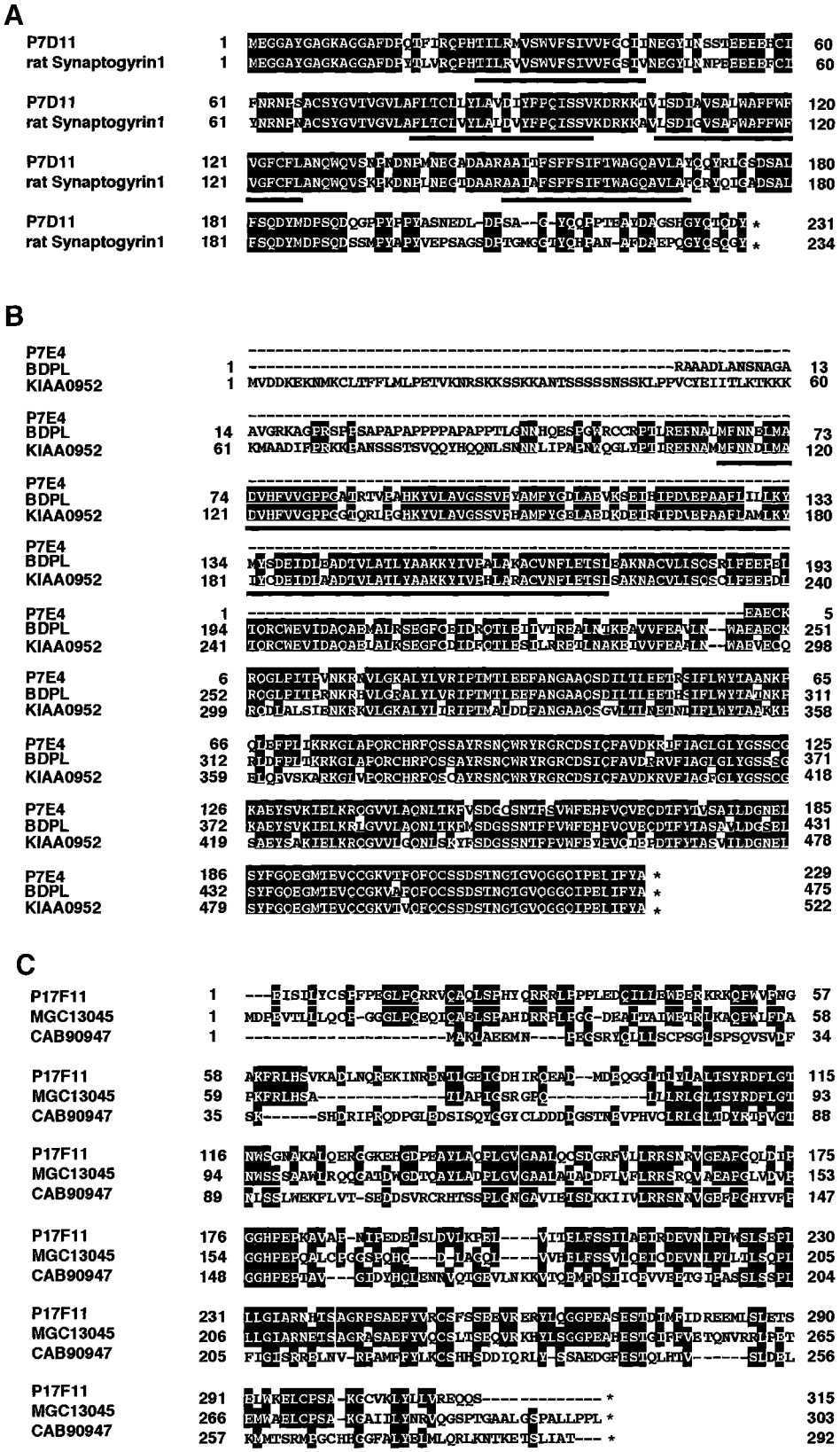
Construction and EST Screening of an AEM cDNA Library

The AEM region, which includes the prechordal plate and anterior endoderm, was dissected from about 600 *Xenopus* embryos from the late gastrula to early neurula stages (stages 12.5–13) (Fig. 1A). During these stages, the morphological differentiation of the head organizer (AEM) and the trunk organizer (notochord) takes place (Nieuwkoop and Faber, 1967), and several known organizer genes have a peak or maintain high level of transcripts as is seen in the case of *Xlim-1* (Taira *et al.*, 1992), *cerberus* (Bouwmeester *et al.*, 1996), *chordin* (Sasai *et al.*, 1994), or *frzb-1* (Wang *et al.*, 1997a). Approximately 180 μg of total RNA was obtained from the collected pieces, and we therefore estimate that we obtained approximately 0.3 μg RNA per a dissected AEM. Since 5–6 μg of total RNA is typically isolated from a whole embryo, it was expected that a roughly 20-fold enrichment of organizer-specific genes would be produced by the dissection. To verify the purity of dissected AEM tissue, a Northern blot with RNAs of the AEM, ANE, and the carcass was hybridized with probes of the organizer-specific genes, *cerberus* (Bouwmeester *et al.*, 1996), *XHex* (Newman *et al.*, 1997), and *crescent* (Shibata *et al.*, 2000), sequentially. As shown in Fig. 1B, intense signals of *cerberus*, *XHex*, and *crescent* were detected in the AEM but not in the ANE, indicating that dissection of the AEM was performed adequately. As for *cerberus*, the signals in the AEM and the carcass were of almost equal intensity, probably because the expression domain of *cerberus* extends laterally to the area including the cardiac primordium (Bouwmeester *et al.*, 1996).

Using poly(A)$^+$ RNA of the AEM, a head organizer-specific cDNA library was constructed. To eliminate abundantly and posteriorly expressed genes, and housekeeping genes from this cDNA library, plaque hybridization was performed by using labeled cDNAs synthesized with RNA of the trunk region posterior to the otic vesicle, which was isolated from stage-26 tailbud embryos. Of 3265 clones plated, 926 (28%) were strongly positive and represent either genes enriched in the trunk region, or those that are ubiquitously highly expressed in the whole embryo. Of 2339 negative clones, we randomly picked 1632 clones which were expected to represent genes enriched in the AEM. Inserts of clones were amplified by PCR with vector primers, and sequenced from the 5′ end. Sequencing from the 3′ end using purified PCR products as templates did not work well, even though several types of primers were designed and examined. This may be due to the variable number of A's at the 3′ end of PCR fragments as a result of a PCR artifact. Of 1632 clones, 1039 were informative and used to construct a local EST database (named the AEM 5′-ESTs), whereas the remaining 593 clones were not, mainly due to extremely short inserts or contamination with other inserts.

To eliminate redundant clones, clustering analysis was performed for 1039 clones of the AEM 5′-ESTs, revealing 754 (73%) putative unique clones. Of 754 putative unique clusters, 608 (80%) were nonredundant clusters (only one clone was contained in a cluster). It should be noted that the number of 754 clusters may be reduced when complete sequences of clones are used. As shown in Fig. 2A, the frequency of clusters decreased rapidly as the number of clones in each cluster increased. The most redundant

FIG. 4. Alignments of the deduced amino acid sequences of isolated clones and proteins similar to each clone. (A) P7D11 and rat Synaptogyrin 1 (Accession No, NP 062039). P7D11 was predicted to contain a full coding region. Four transmembrane domains are underlined. (B) P7E4 and human hypothetical proteins, the BTB domain protein BDPL (Accession No. AAK39520) and KIAA0952 (Accession No. NP 055777). P7E4 lacks the 5′-region. BTB domains conserved between BDPL and KIAA0952 proteins are underlined. (C) P17F11, a human hypothetical protein, MGC13045 (Accession No. NP 115720), and an *Arabidopsis thaliana* putative protein (Accession No. CAB90947), referred as to CAB90947. P17F11 seems to lack the 5′-region. Boxes, identical residues; asterisk, stop codon. Numerical figures on P7D11, rat Synaptogyrin 1, BDPL, KIAA0952, MGC13045, and CAB90947, and indicate amino acid positions starting with the predicted initiation methionine. Numerical figures on P7E4 and P17F11 indicate amino acid positions starting with the N-terminal-most residue.



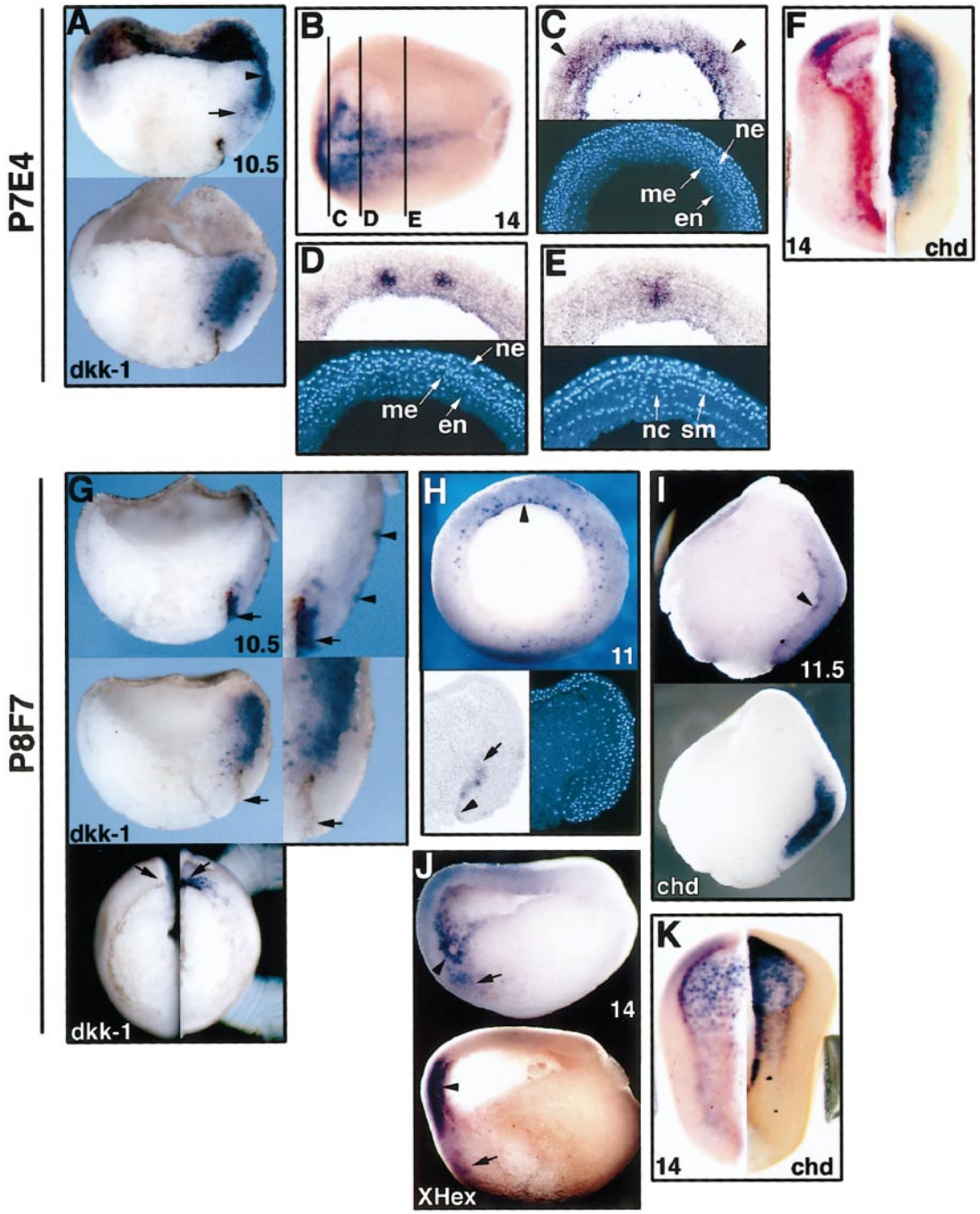
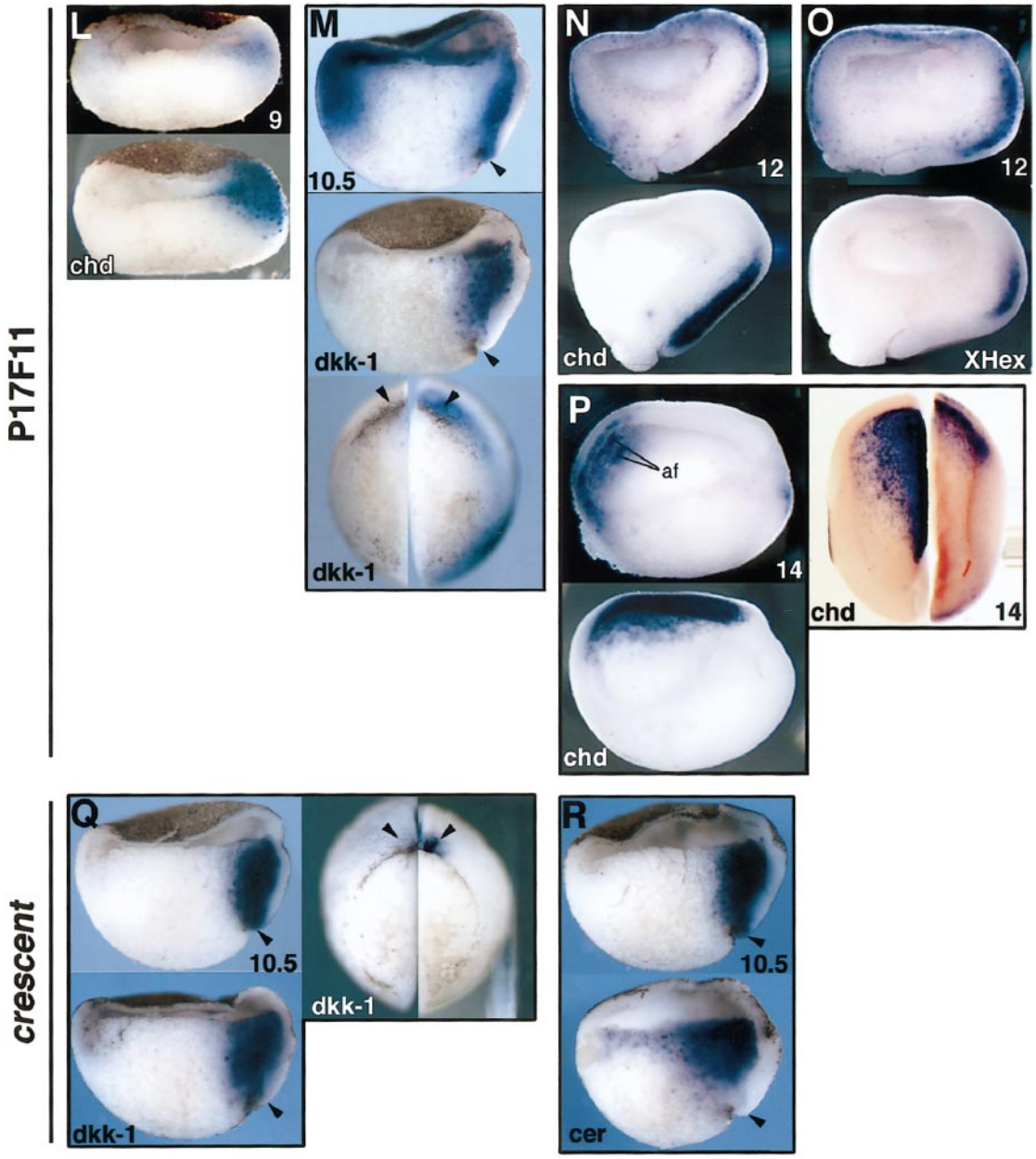


FIG. 5. Detailed expression pattern analysis of P7E4, P8F7, and P17F11 by whole-mount *in situ* hybridization. Whole or hemisectioned embryos were hybridized with P7E4 (A–F), P8F7 (G–K), P17F11 (L–P), or *crescent* (Q, R) probe. Other halves of hemisectioned embryos were used for *dkk-1*, *cerberus* (*cer*), *chordin* (*chd*), or *XHex* probe as indicated. Stained embryos were treated with BB/BA to become transparent for observation (B, F, K, P, right panel). Sections were made from stained whole embryos (C–E, H), and are shown in bright field and with DAPI staining. (A–F) P7E4 (homologous to human hypothetical proteins, BDPL and KIAA0952). (A) Sagittal hemisections of early gastrulae (stage 10.5) stained for P7E4 (upper panel) and *dkk-1* (lower panel). P7E4 expression was detected around the Brachet's cleft (arrowhead) and weakly in the mesoderm region (arrow). Dorsal to the right. (B) Dorsal view of early neurula. Anterior to the left. Cross sections were made at the indicated positions (C–E) which correspond to the following panels (C–E). (C–E) Sections of the same embryo as shown in (B). P7E4 was expressed in the archenteron roof as well as the bilateral domains including the neuroectoderm and mesoderm region (arrowheads) (C). The bilateral expression domains were notable in the mesodermal region at position D. In the posterior region, expression was seen in the notochord (E). (F) Dorsal view of sagittally hemisectioned neurula embryos stained for P7E4 and *chordin*. Anterior is up. (G–K) P8F7 (unknown). (G) Sagittal hemisections of early gastrula stained for P8F7 (top) and *dkk-1* (middle), and vegetal view



of the hemisectioned embryo (bottom). Right panels, higher magnification of the left panels. Dorsal is to the right (top, middle) or up (bottom). Arrow, the surface layer of the dorsal blastopore lip, expressing P8F7 but not dkk-1; arrowhead, punctuated expression of P8F7 in the epithelial layer of the dorsal ectoderm. (H) Vegetal view of early gastrula (upper panel, dorsal is up) and a sagittal section (lower panels, dorsal to the right). Arrowhead, dorsal blastopore lip; arrow, anterior archenteron. (I) Sagittal hemisections of midgastrula stained for P8F7 and *chordin*. Dorsal to the right. Arrowhead, expression of P8F7 in the anterior archenteron roof. (J) Sagittal hemisections of early neurula stained for P8F7 and *XHex*. Anterior to the left. Arrow, expression in the anterior endoderm; arrowhead, expression in the anterior archenteron (P8F7) or the prechordal plate (*XHex*). (K) Dorsal view of sagittally hemisectioned neurula stained for P8F7 and *chordin*. Anterior is up. (L–P) P17F11 (related to human MGC13045 and *Arabidopsis* CAB90947). (L) Sagittal hemisections of late blastula stained for P17F11 and *chordin*. (M) Sagittal hemisections of early gastrula stained for P17F11 (top) and *dkk-1* (middle), and vegetal view of the hemisectioned embryo (bottom). Dorsal is to the right (top, middle), or up (bottom). Arrowhead, the surface layer of dorsal blastopore lip, expressing P17F11 but not *dkk-1*. (N, O) Sagittal hemisections of late gastrulae stained for P17F11, *chordin*, or *XHex*. Dorsoanterior to the right. (P) Sagittal hemisections of early neurula stained for P17F11 (upper) and *chordin* (lower), and dorsal view of the hemisectioned embryo (right). Partial breakage of the archenteron roof shows interior staining in the anterior mesoderm as indicated by af. Anterior is to the left (left panels), or up (right panel). (Q, R) *crescent*. (Q) Sagittal hemisections of early gastrula stained for *crescent* (upper) or *dkk-1* (lower), and vegetal view of the hemisectioned embryo (right). Dorsal is to the right (left panels), or up (right panel). (R) Sagittal hemisections of early gastrula stained for *crescent* and *cerberus*. Dorsal is to the right. Arrowheads, the surface layer of dorsal blastopore lip, expressing *crescent* but not *dkk-1* or *cerberus*. Numerical figures in the panels indicate the developmental stages according to Nieuwkoop and Faber (1967). Pictures of hemisections stained for *dkk-1*, *cerberus*, *chordin*, and *XHex* were flipped for comparison. af, artifact; en, endoderm; nc, notochord; ne, neuroectoderm; sm, somitic mesoderm.

TABLE 2
Summary of Isolated cDNA Clones Expressed in the Organizer Region

Clone name	No. of times isolated	Insert (bp)	mRNA (kb)	Homology search					
				Identity				Score	E. value
P4E7	1	1262	3.3	blastn	BG346293	*X. laevis*	EST	815	0
P5A5	1	732	1.4	blastn	BE189015	*X. laevis*	EST	821	0
P7D11	1	1337	1.5	blastx	NP_062039	*R. norvegicus*	synaptogyrin 1	351	1E-95
P7E4	1	1134	3.5/2.8	blastx	AAK39520	*H. sapiens*	BTB domain protein (BDPL)	434	1E-120
				blastx	NP_055777	*H. sapiens*	hypothetical protein KIAA0952	201	7E-94
P8F7	1	1447	2.2	blastn	AW633257	*X. laevis*	EST	616	1E-175
P11F3	1	291	<0.66	blastn	AW147620	*X. laevis*	EST	474	1E-133
P16H6	1	572	3.4	blastn	BE132299	*X. laevis*	EST	266	2E-70
P17F11	2	1051	1.4	blastx	NP_115720	*H. sapiens*	hypothetical protein MGC13045	222	5E-65
				blastx	CAB90947	*A. thaliana*	hypothetical protein	98	2E-19

Note. Isolated clones expressed in the organizer region are listed. mRNA sizes were deduced from Northern blot analysis (Fig. 3B). Of results of homology search obtained by the blastn, blastx, and tblastn programs, the one with the smallest E-value less than 1E-15 is listed in each clone. In P7E4 and P17F11, human KIAA0952 and *Arabidopsis* genes are also listed, showing significant similarity. As for the genes that have no significant homology with genes in public databases (GenBank + EMBL + DDBJ + PDB or GenBank CDS translations + PDB + SwissProt + PIR + PRF; search date, June 20, 2001), the results of comparison with public *Xenopus* ESTs are shown. <, The accurate size could not be determined by conventional Northern blot hybridization because of small mRNA size. DDBJ/Genbank/EMBL Accession Nos. are as follows: P4E7 (AB072008), P5A5 (AB072004), P7D11 (AB072005), P7E4 (AB072006), P8F7 (AB072007), P11F3 (AB072001), P16H6, (AB072003), and P17F11 (AB072002).

clones were *Xcirp* (encoding cold-inducible RNA binding protein) (Uochi and Asashima, 1998) (isolated 39 times), glutamine synthetase (Hatada *et al.*, 1995) (13 times), and a putative splicing factor, PR264 (Vellard *et al.*, 1992) (11 times). The putative unique 754 clusters were classified according to their similarity to other genes in a public database (GenBank Release 119 Oct/2000) by using the blastn program. As shown in the left column of Fig. 2B, 20% (151) of 754 unique clones represented known *Xenopus* genes, whereas 56% (420) showed no similarity to other genes.

To determine the isolation frequency of known organizer genes, we attempted to list the isolated known *Xenopus* organizer genes as well as the *Xenopus* genes which are reported to show highly regionalized expression patterns (Table 1). The isolation frequency of organizer genes estimated here may reflect that of novel organizer genes expected to be isolated by expression pattern screening, as described below. Of the 151 known *Xenopus* genes, 14 (9%) are reportedly expressed in the organizer region, and 8 (5%) of them were organizer-specific. Those include genes encoding secreted molecules, the inhibitory factors such as BMP-antagonists Chordin (Piccolo *et al.*, 1996), Noggin (Zimmerman *et al.*, 1996), Follistatin (Iemura *et al.*, 1998), and possibly xFRP1 (Okabayashi *et al.*, 1999). Genes reported to be expressed mainly in the neural plate and other regions such as lateral mesoderm and endoderm were also found in the cDNA library (3.3 and 4.0%, respectively, of 151 known *Xenopus* genes; Table 1), implying either contamination of those tissues in our AEM preparation or possible expression of those genes in the AEM region, or both.

To select clones for the expression pattern screening, functional classification was also attempted based on homologies to known definitive or putative proteins in protein databases (all nonredundant GenBank CDS translations Release 119 Oct/2000; SwissProt Release 39 Nov/2000; PIR/PDB Release 67 Sep/2000) by using the blastx program (Fig. 2B, middle column). Of 754 unique clones, 11% (84) represented genes with predicted or established functions as developmental regulators, whereas 42% (315) were unclassified. These unclassified clones included genes encoding proteins with no similarity to other proteins or with unestablished functions. According to the functional classification, 354 unique clones, which were either functionally unclassified or developmental regulators of non-*Xenopus* species, were selected for a whole-mount *in situ* hybridization screen.

Expression Pattern Screening of an AEM Library

Of 354 selected clones, 198 arbitrarily selected clones were analyzed for expression patterns with various stages of embryos as described in Materials and Methods, as well as blastula (stage 9) and gastrula (stages 10–11) when necessary. As shown in the right column of Fig. 2B, expression patterns were classified into "patterned" (localized expression pattern in at least one of the stages examined), "ubiquitous" (ubiquitous expression throughout the stages examined), and "undetected" (no clear signal throughout the stages examined after staining for 2 days). Of 198 clones,

49% (97) were classified into "patterned," and 9 clones including *Xenopus* crescent as previously reported (Shibata *et al.*, 2000) were found to be apparently expressed in organizer-derived tissues (anterior endoderm, prechordal plate, and notochord) at the early neurula stage. These clones were isolated with a frequency of 6% (9 out of 198), which is similar to the frequency predicted above in the case of known *Xenopus* organizer-specific genes (5%; 8 out of 151). Figure 3A shows the expression patterns of eight isolated clones (the expression pattern of *Xenopus* crescent, which has been published, is not included). These eight clones were fully sequenced on both strands, and the entire sequences were compared against public DNA and protein databases as described below.

To examine mRNA sizes and temporal expression patterns, Northern blot hybridization was carried out by using total RNA from four-cell (stage 3) to tailbud (stage 28) stage embryos for *crescent*, or from late blastula (stage 9) to midneurula stage embryos (stage 15) for other genes (Fig. 3B). Three patterns of developmental expression were observed most frequently: (1) levels of expression gradually increase from stage 10 or 12.5 onward (P4E7, P8F7, and P11F3); (2) high levels of expression are seen at stage 9, then gradually decrease (P5A5, P7D11, and P16H6); and (3) expression peaks at stages 10–12.5 (P17F11). Interestingly, with P7E4, the transcripts of 3.5 kb at late blastula (stage 9) decreased at stage 10, whereas a zygotic mRNA of 2.8 kb, in turn, started to be expressed at this stage. The size difference between the mRNAs is probably due to alternative splicing or cross-hybridization to a related gene.

The entire sequences of these clones were compared against the nonredundant (nr) DNA database (NCBI) and the *Xenopus* ESTs using blastn, and the protein databases (all nonredundant GenBank CDS translations + PDB + SwissProt + PIR + PRF) by using blastx. In addition, each of the translated amino acid sequences from the three reading frames was used in a search against the same DNA database by using tblastn. Of the eight genes, three clones named P7D11, P7E4, and P17F11 showed similarities to other known or putative genes. P7D11 contained a complete open reading frame, and its translated amino acid sequence showed 77% identity to rat Synaptogyrin 1 (Fig. 4A), a member of the synaptic protein family (Stenius *et al.*, 1995). P7E4 and P17F11 were similar to hypothetical proteins. Though P7E4 seemed to lack the 5′ region of its open reading frame, the remaining region, when translated into amino acid sequence, showed 92 and 81% identities to human hypothetical proteins, a BTB domain protein (named BDPL; Accession No. AAK39520) and KIAA0952 (Accession No. NP 055777), respectively (Fig. 4B). A translated P17F11 sequence showed 52 and 31% identities to a human hypothetical protein MGC13045 (Accession No. NP 115720) and an *Arabidopsis thaliana* putative protein (Accession No. CAB90947), respectively. According to this alignment, P17F11 seemed to lack the 5′ region of its open reading frame (Fig. 4C). The remaining five genes, P4E7, P5A5, P8F7, P11F3, and P16H6, did not have any similarity to other genes in the databases except the *Xenopus* ESTs that were also not similar to any other known genes. Table 2 summarizes results of the homology search as well as mRNA size and insert length for the eight isolated clones except *crescent*.

Expression of P7E4, P8F7, and P17F11 in the AEM Region

Of the eight isolated genes, P7E4, P8F7, and P17F11 were expressed notably in the anterior part of the embryo (Fig. 3A). To analyze their detailed expression patterns and to confirm that these genes are expressed in the AEM, we carried out whole-mount *in situ* hybridization in comparison with four known organizer genes: *dkk-1* and *cerberus* which mark the prospective prechordal plate (Glinka *et al.*, 1998) and the anterior endoderm and prospective prechordal plate (Bouwmeester *et al.*, 1996), respectively, at the early gastrula stage (stage 10.5), and *chordin* and *XHex* which mark the prechordal plate and notochord (Sasai *et al.*, 1994) and the anterior endoderm and prechordal plate (Jones *et al.*, 1999), respectively, at midgastrula to early neurula stages (stage 12–14).

At the early gastrula stage (stage 10.5), P7E4 (a homolog of the human BDPL and KIAA0952 genes) was expressed in the entire ectoderm layer, notably along the Brachet's cleft (Fig. 5A, arrowhead in the upper panel). This gene was also expressed in the dorsal mesoderm including the prospective prechordal plate (Fig. 5A, arrow), where *dkk-1* was expressed (Fig. 5B, lower panel). At the early neurula stage (stage 14), a localized expression pattern of P7E4 was observed in the anterior region and along the midline in the trunk region (Fig. 5B). Cross sections showed that in the anterior region P7E4 was expressed in the endodermal cells covering the archenteron, and bilaterally in the neuroectoderm and mesoderm region (Fig. 5C, arrowheads). This mesoderm region is probably within the prechordal plate. In the more posterior part of the embryo, the bilateral expression domains of P7E4 were clearly observed in the mesoderm layer (Fig. 5D) and was finally confined to a single region corresponding to the notochord (Fig. 5E). When viewed dorsally at the neurula stage (stage 14), anterior expression of P7E4 encircles the region that appears to include the prechordal plate where *chordin* is expressed (Fig. 5F).

Localized expression of P8F7 (an unclassified gene) was faintly detected at the early gastrula (stage 10.5) in the ectoderm (Fig. 5G, arrowheads in the upper right panel), and more intensely in the involuting surface layer of the dorsal blastopore lip which corresponds to the prospective pharyngeal endoderm (arrows in the top and bottom panels). In contrast, the expression of the head organizer gene *dkk-1* was detected in the dorsal endoderm and mesoderm but not in the surface layer of the dorsal blastopore lip (Fig. 5G, arrows in the middle and bottom panels). A vegetal view showed a punctuated expression pattern of P8F7 along the blastopore lip with a dorsolateral gradient (Fig. 5H, upper

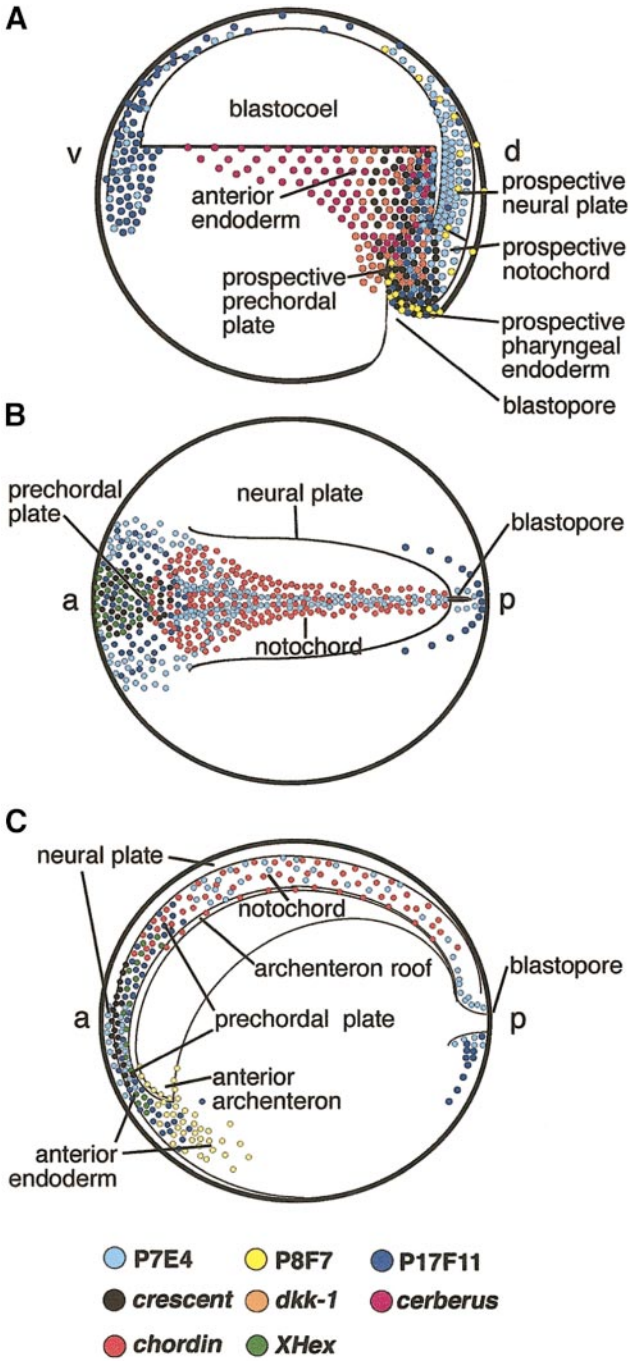


FIG. 6. Summary of expression area s of isolated clones in comparison with *dkk-1*, *cerberus*, *chordin*, and *XHex*. (A) Sagittal section view of the early gastrula (stage 10.5). The expression domain of known organizer genes, *dkk-1* and *cerberus*, were indicated for comparison. (B) Dorsal view of the early neurula (stage 14). The expression domain of known organizer genes, *chordin* and *XHex*, were indicated for comparison. (C) Sagittal section view of the early neurula (stage 14). The expression domain of *crescent* at the neurula stage in (B) and (C) were deduced from Shibata *et al.* (2000). Expression domains of each gene are represented by colored dots: light blue, P7E4 (related to human BDPL and KIAA0952); yellow, P8F7 (unknown); blue, P17F11 (related to human MGC13045 and *Arabidopsis* CAB90947); black, *crescent*; red, *chordin*; green, *XHex*; purple, *cerberus*; magenta, dkk-1. d, dorsal; v, ventral; a, anterior; p, posterior.

panel). Sagittal sections of the midgastrula (stage 11) showed that the signal was detected in the archenteron roof (Fig. 5H, arrow in the lower panel). Sagittally hemisectioned embryos at stage 11.5 showed a signal in the anterior archenteron roof (Fig. 5I, arrowhead in the upper panel), the posterior part of which overlapped with the prechordal and chordal expression of *chordin* (Fig. 5I, lower panel). In addition, P8F7 appeared to be expressed as a broad signal in the three germ layers of the dorsoanterior region (Fig. 5I, upper panel; see also Fig. 5J, upper panel). At the early neurula stage (stage 14), P8F7 expression was still seen in the anterior archenteron and the anterior endoderm (Fig. 5J, arrowhead and arrow, respectively, in the upper panel). In contrast, *XHex* was expressed strongly in the prechordal plate and less strongly in anterior endoderm (Fig. 5J, arrow and arrowhead, respectively, in the lower panel). When viewed dorsally at the neurula stage, the expression of P8F7 was most intensely detected in the anterior archenteron with a punctuated pattern (Fig. 5K).

Transcripts of P17F11 (similar to human MGC13045 and *Arabidopsis* CAB90947) were detected at the late blastula stage (stage 9) in the dorsal blastocoel floor and dorsal marginal zone that was included by the domain of *chordin* expression (Fig. 5L). At the gastrula stage (stage 10.5), P17F11 expression was detected mainly in the dorsal mesoderm and endoderm, as well as in the ventral ectoderm and mesoderm (Fig. 5M). Compared to *dkk-1* and *cerberus*, P17F11 was expressed in a more shallow layer on the dorsal side. In addition, the signal in the leading edge of the involuting endoderm was weaker than that of *dkk-1* (Fig. 5M) and *cerberus* (not shown). Unlike *dkk-1*, P17F11 was expressed in the surface layer of the dorsal blastopore lip (Fig. 5M, arrowheads), and the expression domain of P17F11 extended more laterally than those of *dkk-1* (Fig. 5M, bottom panel) and *cerberus* (not shown). At the late gastrula stage (stage 12), P17F11 expression was seen in an AEM region, which overlapped with the anterior region of *chordin* expression (Fig. 5N), and covered the expression domain of *XHex* (Fig. 5O). In the ectodermal region, broad expression of P17F11 was also observed on the ventral side (Figs. 5N and 5O). At neurula stages, P17F11 expression was detected in the AEM, and still overlapped with the anterior region of *chordin* expression (Fig. 5P).

Though the developmental expression pattern of *crescent/frzb2* has been previously reported by other groups and us (Bradley *et al.*, 2000; Pera and De Robertis, 2000; Shibata *et al.*, 2000), we reexamined the expression domain of *crescent* in comparison with those of *dkk-1* and *cerberus* at the early gastrula stage (stage 10.5). The expression

domain of *crescent* in the AEM was narrower than those of *dkk-1* and *cerberus* (Figs. 5Q and 5R), and, unlike *dkk-1* and *cerberus*, *crescent* was expressed in the surface layer of the dorsal blastopore lip (Figs. 5Q and 5R, arrowheads). Figure 6 summarizes the expression domains of P7E4, P8F7, P17F11, and *crescent*, as well as *dkk-1*, *cerberus*, *chordin*, and *XHex* at the early gastrula and early neurula stages. These results indicate that the genes isolated in this study as well as the known organizer genes have distinct expression domains in the AEM region at gastrula to neurula stages.

DISCUSSION

Systematic Screening for Head Organizer Genes

The head organizer region corresponds to the AEM, which includes the anterior endoderm and the prechordal plate, in gastrula to early neurula *Xenopus* embryos. To prepare for a systematic screen that will allow us to analyze the molecular and functional complexity of the head organizer, we constructed an AEM cDNA library using dissected embryos to increase the efficiency of isolating region-specific genes. The degree of enrichment of head organizer genes by dissection was estimated by following *Xenopus* crescent, which was isolated in this screening as follows. In our EST screening, the *crescent* gene appeared twice in 1039 randomly picked clones (the probability is about 2×10^{-3}) (Table 1), whereas screening of a midgastrula cDNA library using this isolated clone as probe yielded 53 positive plaques from 8×10^5 plaques (the probability is about 7×10^{-5}) (Shibata *et al.*, 2000). Thus, an approximate 30-fold enrichment of the *crescent* gene is estimated. This value is in good agreement with the initial estimation based on the ratio of amounts of RNA extracted from AEM to that from whole embryos, which we calculated to be approximately 1:20 (see above).

To remove highly expressed genes, we adopted negative selection by plaque hybridization with a cDNA probe prepared from the trunk region, and constructed ESTs before the expression pattern screen was performed by whole-mount *in situ* hybridization. As a result of plaque hybridization, we removed 28% of the clones which are expected to be highly redundant genes expressed in the trunk region. Nevertheless, highly redundant clones do exist in the ESTs, such as *Xcirp* which appeared 39 times in a single cluster (maximum redundancy among all clusters in the ESTs) and shared approximately 4% of all picked clones (1039 clones). This suggests that the plaque hybridization that we carried out was effective to eliminate the genes that account for more than 4% of total clones.

Of 1039 EST clones (754 clusters), we isolated 8 known organizer-specific genes, including *chordin*, *noggin*, *follistatin*, and *XHex*. To our surprise, the majority of the reported organizer genes, except *Xlim-1*, *goosecoid*, and *Xotx2*, were identified in just about 1000 ESTs. It is noteworthy that the 13 clusters with more than 6 clones in our random screen were all known genes (not shown), including *Xcirp*, glutamine synthetase, and a putative splicing factor, PR264, which were previously isolated by various approaches and techniques. This implies that relatively highly expressed genes are easily isolated no matter what screening protocol is followed.

The introduction of the generation of ESTs into the gene screening protocol also provides a clue about the molecular composition of the organizer. The molecules secreted from the organizer can be divided into "inhibitory factors" and "ligand molecules." The former category includes BMP inhibitors (Chordin, Noggin, Follistatin, and Cerberus) (Iemura *et al.*, 1998; Piccolo *et al.*, 1999; Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996), Wnt inhibitors (Frzb-1, Dkk-1, Cerberus, and putatively Crescent) (Bradley *et al.*, 2000; Glinka *et al.*, 1998; Lin *et al.*, 1997; Pera and De Robertis, 2000; Piccolo *et al.*, 1999; Wang *et al.*, 1997b), and Nodal inhibitors (Cerberus and Lefty/Antivin) (Cheng *et al.*, 2000; Piccolo *et al.*, 1999). The latter includes Xnr1, Xnr2, Xnr4 (Jones *et al.*, 1995; Joseph and Melton, 1997; Smith *et al.*, 1995), eFGF (Isaacs *et al.*, 1995), Xwnt-11 (Ku and Melton, 1993), and Sonic hedgehog (Shh) (Ekker *et al.*, 1995), which are supposed to bind to specific receptors and activate intracellular signal cascades. As shown in Table 1, 4 genes in the 151 *Xenopus* clusters encode organizer-specific secreted molecules, all of which are inhibitory factors (*chordin* $\times$ 3, *noggin* $\times$ 1, *follistatin* $\times$ 1, *xFRP* $\times$ 1), not ligand molecules. These data implies that, if the frequency of a given cDNA reflects the expression level of the corresponding mRNA, the inducing molecules secreted from the organizer are predominantly inhibitory factors. It might also be interesting to compare the gene variety and frequency between organizer-specific transcriptional activators such as Xlim-1 (Breen *et al.*, 1998; Hiratani *et al.*, 2001), Otx2 (Gammill and Sive, 1997), XFKH1/Pintallavis/HNF-3β (Pani *et al.*, 1992), and Xbra (Tada *et al.*, 1998), and transcriptional repressors such as Goosecoid, (Artinger *et al.*, 1997), Xanf-1/Rpx/Hesx (Ermakova *et al.*, 1999), XHex (Brickman *et al.*, 2000), and Xblimp1 (de Souza *et al.*, 1999). However, compared to secreted molecules, genes for transcription factors were isolated with much less frequency, as genes encoding the transcription repressor XHex and the activator Xbra were isolated only once each. This is probably due to the low levels of expression of transcription factors as compared to those of secreted molecules. Further screens may provide insight into the composition of transcripts for transcription factors.

A gene expression pattern screen of *Xenopus* embryos by whole-mount *in situ* hybridization was first reported by Gawantka *et al.* (1998), and resulted in the isolation of several developmental regulators, such as *Xvent-1* (Gawantka *et al.*, 1995), *Xvent-2* (Onichtchouk *et al.*, 1996), *BAMBI* (Onichtchouk *et al.*, 1999), and *Xblimp-1* (de Souza *et al.*, 1999). In their approach, clones from a cDNA library of whole gastrula embryos were randomly picked and directly analyzed by whole-mount *in situ* hybridization to obtain an overview of global gene expression patterns in early embryogenesis. Partial sequence analysis and cluster-

ing were carried out only for the differentially expressed genes, in order to describe which genes had been identified. As for head organizer genes, 4 were isolated by this approach (*Xanf-1*, *Xotx2*, *frzb-1*, and *Xblimp-1*) from 1765 clones examined (Gawantka *et al.*, 1998). In our study, we focused instead on the effective isolation of genes expressed in a particular region, the AEM. Partial sequencing and clustering of clones from the AEM cDNA library were carried out first, followed by expression pattern analyses conducted only for selected genes. As expected, we were able to isolate 4 AEM-specific genes out of 198 clones with an approximately 10-fold higher efficiency at the step of expression pattern screening, even after eliminating known *Xenopus* genes.

Expression Analysis of AEM-Specific Genes

A comparison of the expression domains of P7E4, P8F7, and P17F11, as well as *crescent* with those of known organizer genes such as *dkk-1*, *cerberus*, *chordin*, and *XHex* revealed that these clones show distinct expression patterns which demarcate the domains in the AEM, which corresponds to the head organizer region (Figs. 5 and 6). The previously isolated organizer genes have been shown to subdivide the AEM roughly into the anterior endoderm and the prechordal plate during neurula stages and appear to play some roles peculiar to each domain. For example, *XHex*, a gene expressed in the anterior endoderm, encodes a transcriptional repressor which has been suggested to define the anterior-most limit of the organizer, possibly by repressing *goosecoid* and *chordin* (Jones *et al.*, 1999) and by indirectly upregulating *cerberus* (Zorn *et al.*, 1999).

As P8F7 is expressed in the surface layer of the dorsal blastopore lip, which includes a region possessing the organizer activity (Shih and Keller, 1992), at the early gastrula stage, and later in the anterior portion of archenteron, at the early neurula stage (Figs. 5 and 6), this gene is implicated in playing a role in the organizer activity as well as specifying the pharyngeal endoderm. On the specification of prechordal plate, a model has been proposed recently (Mochizuki *et al.*, 2000) that the autoinhibitory action of Goosecoid is counteracted by a high level of Xotx2, whose expression is maintained by Xlim-1 plus Ldb1, and Goosecoid in turn suppresses *Xbra* expression (Artinger *et al.*, 1997). Of genes isolated in our screen, P7E4 and P17F11 were found to be expressed in the prechordal plate, though these genes are broadly expressed in mesoderm and ectoderm regions at gastrula stages (Figs. 5 and 6). P7E4 encircles the anterior region including prechordal plate and is also expressed in the notochord; P17F11 is expressed widely in the AEM. To our knowledge, the expression patterns of P7E4 and P17F11 do not resemble those of any reported genes. Thus, it is expected that these genes may play novel roles in the specification of the head organizer, neural induction, or patterning in each area.

Screening by expression pattern analysis has the advantage of potentially revealing new expression domains of genes whose expression patterns have been examined in other species. Clone P7D11 is predicted to contain a complete open reading frame that was highly related to the genes encoding rat Synaptogyrin 1 (77% identity of amino acid sequence). Synaptogyrin 1 is a member of a family of abundant synaptic vesicle proteins (Baumert *et al.*, 1990). Recently, it was reported that Synaptogyrin 1 is potent inhibitor of exocytosis (Sugita *et al.*, 1999). In rat tissue, Synaptogyrin 1 is found in nerve terminals associated with small synaptic vesicles and, outside the nervous system, found only in endocrine cell types specialized for peptide secretion (Baumert *et al.*, 1990). The expression pattern in murine development is not known, but, unexpectedly, it was revealed that transcripts for Synaptogyrin 1 are specifically expressed in the notochord in the *Xenopus* early neurula (Fig. 3A). It is likely that in *Xenopus* early development, Synaptogyrin 1 may regulate the secretion of some peptides from the notochord. Clone P7E4 and P17F11 were similar to putative genes encoding human BDPL and KIAA0952, and human MGC13045 and *Arabidopsis* CAB90947, respectively. These are all functionally unknown proteins, and the isolation of the full-length cDNAs, and the determination of their gene structure needs to be studied further.

In this study, we present data indicating that a screening strategy including the use of ESTs data are effective for isolating novel organizer-specific genes. Future studies on their functions will provide insights into the mechanisms of organizer specification and on the functions of the organizer.

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Note added in proof. Recent blast search revealed that putative proteins of P5A5, P8F7, P11F3, and P16H6 were found to be homologous to those of mouse genes (Accession Nos. BAB28303, BAB26499, and AAH02177) and a *Drosophila* gene (Accession No. AAD38615), respectively. Expression patterns of genes examined in this study are available at http://xextdb.nibb.ac.jp.

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