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Maternal Transcripts of Mitotic Checkpoint Gene, *Xbub3*, Are Accumulated in the Animal Blastomeres of *Xenopus* Early Embryo

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ABSTRACT

Maternally transcribed mRNAs play the important role during early embryogenesis. Especially in patterning, distribution of the maternal transcripts has a causal relation to axis formation in the early embryo. We compared the quantity of mRNAs among four blastomeres of *Xenopus* 8-cell-stage embryos by the differential display method. A novel gene, *Xbub3*, was cloned by screening the oocyte cDNA library with an animal blastomere-enriched PCR product. *Xbub3* is a homolog of the human mitotic checkpoint gene *hBub3*. A transcript of *Xbub3* was 2940 bp and encoded a predicted protein of 330 amino acids with six WD repeats. Expression of *Xbub3* was observed from oocyte to tadpole. Whole-mount *in situ* hybridization showed that *Xbub3* mRNAs were uniformly distributed in the early stages of oogenesis but gradually localized to the animal hemisphere, especially in the perinuclear cytoplasm of full-grown oocytes. In the cleavage-stage embryos, the maternal transcripts of *Xbub3* were recruited into each blastomere, associating closely with chromosomes. Zygotic expression of *Xbub3* was widely detected in gastrula ectoderm and was gradually restricted to the central nervous systems, eyes, and branchial arches by the tadpole stage. This evidence contributes to understanding of the regulatory mechanism of the cell cycle and cell differentiation in the early embryo.

INTRODUCTION

IN *XENOPUS* EMBRYOS, the establishment of the anterior–posterior axis has been accomplished during oogenesis. There is a germinal vesicle on the future animal hemisphere and a mitochondrial mass on the future vegetal hemisphere. Other body axes, the dorsal–ventral and the left–right, are triggered by the cortical rotation just after fertilization. The cytoplasmic rearrangement by the cortical rotation causes the dorsal formation from the side opposite the sperm entry site (Elinson, 1980; Vincent *et al.*, 1986). Ultraviolet irradiation and cytoplasm transplantation experiments have suggested that dorsal formation of the embryo depends on the cytoplasmic components which are localized at the vegetal pole before the cleavage stage (Elinson and Pasceri, 1989; Kao and Danilchik, 1991; Fujisue *et al.*, 1993; Sakai, 1996). Isolation and transplantation experiments of the specified blastomeres of 8-cell or 32-cell embryos also have shown that factors important for axis formation are distributed to special blastomeres (Gimlich and Gerhart, 1984; Kageura and Yamana, 1986; Dale and Slack, 1987; Takasaki and Konishi, 1989). It also has been reported that some cytoplasmic factors (Yuge *et al.*, 1990) or mRNAs (Hainski and Moody, 1992) extracted from dorsal blastomeres at the cleavage stage have an ability to form a secondary axis.

Molecular cloning gives us a clue to clarify the cytoplasmic factors which control axis formation in the embryo. Many genes have been reported to have special localization of their transcripts along the animal–vegetal axis of the oocyte. For example, animal localization of transcripts has been observed for An1, encoding ubiquitin-like proteins (Linnen *et al.*, 1993); An2, encoding a subunit of mitochondrial ATPase (Weeks and Melton, 1987a); and BMP-2 (Suzuki *et al.*, 1994; Hemmati-Brivanlou and Thomsen, 1995; Clement *et al.*, 1995). In contrast, Vg1 (Rabegliati *et al.*, 1985; Weeks and Melton, 1987b), Xcat-2 (Mosquera *et al.*, 1993), Xwnt-11 (Ku and Melton, 1993), and Xlsirts (Kloc *et al.*, 1993; Kloc and Etkin, 1994) have vegetal localization of their transcripts in oocytes. Most of these genes have been identified by differential screening methods based on the specific localization of their transcripts. In the present study, 8-cell-stage embryos were separated into

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four groups of blastomeres, and their mRNAs were compared by differential display methods developed by Liang and Pardee (1992).

MATERIALS AND METHODS

Eggs and embryos

Oocytes were obtained by surgical operations from the ovary of adult *Xenopus laevis* females and staged according to Dumont (1972). Fertilized eggs were obtained by injecting 200 to 300 U of human chorionic gonadotropin (Gestron, Denka Seiyaku) into female and male animals. The eggs, dejellied with 1% thioglycolic acid, were reared at 20°C and staged according to Nieuwkoop and Faber (1967). The 8-cell-stage embryos were soaked in phosphate-buffered saline for 2 min at 20°C to loosen the connection among blastomeres. Using fine forceps under a binocular microscope, each of the embryos was separated into four pairs of two corresponding blastomeres from the left and right halves. The separated blastomere pairs were used for RNA extraction.

Differential display method

Total RNAs were extracted by the acid guanidine phenol–chloroform method (Chomczynski and Sacchi, 1987) from the separated blastomere pairs. The first-strand cDNAs were synthesized with oligo(dT) primer and Reversetranscript I (Wako) from 2 μg of total RNA. Single-strand cDNA was

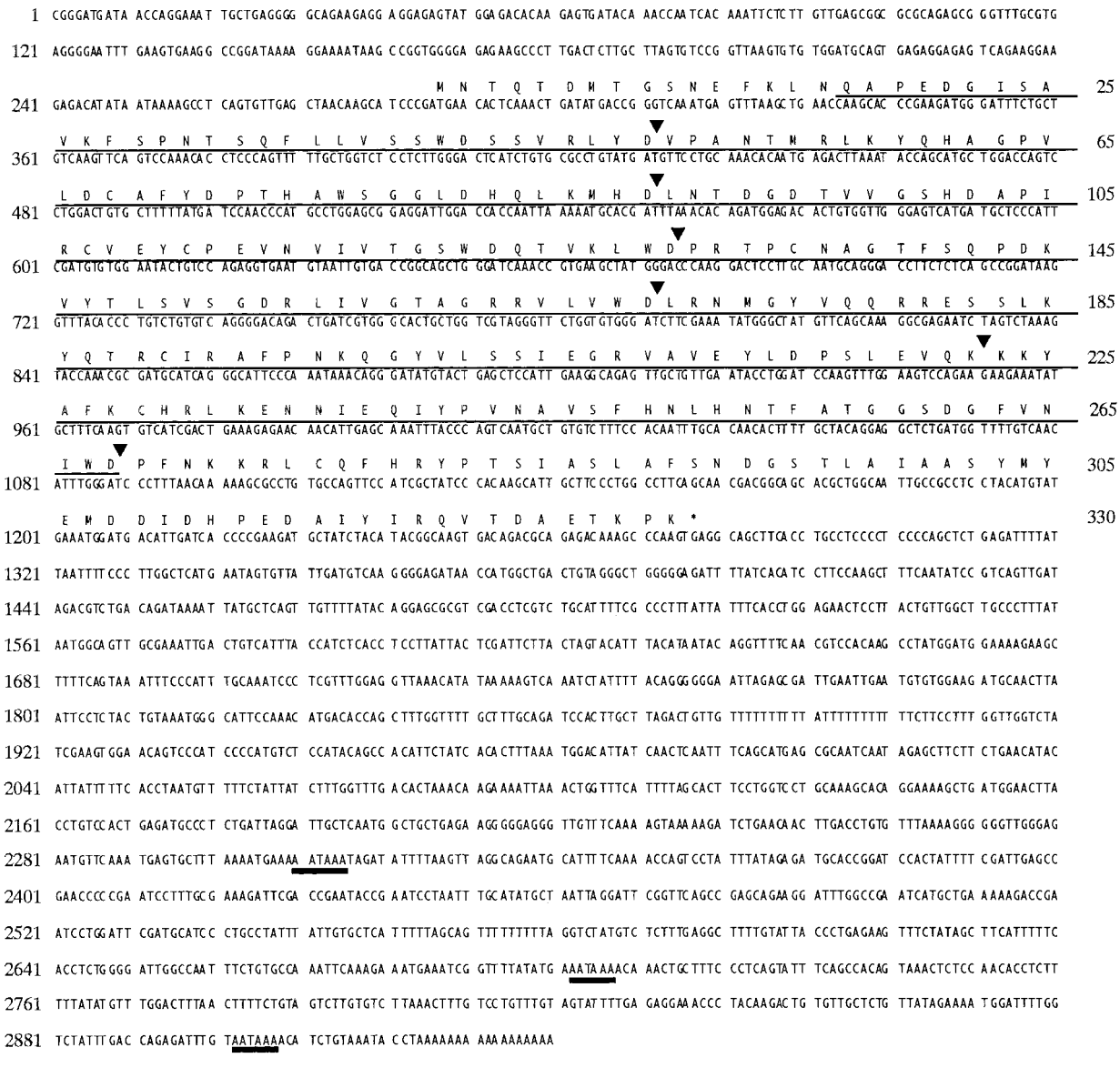

FIG. 1. Nucleotide and protein sequences of *Xbub3*. The 2940-bp cDNA contains a single ORF encoding a predicted protein of 330 amino acids. Arrowheads indicate the presumptive carboxyl end site of each repeat within the WD-repeat motifs (underline). The thick underlines indicate consensus polyadenylation signal sequences.

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Xbub3      MNTQTDMTGSNEFKLNQAPEDGISAVKFSPNTSQFLLVSSWDSSVRLY--DVPANTMR-L   57
hBub3           **********P******S****************T******--*****S**-*   51
Bub3               MQIVQIE***K*Y**DI*II*SK*-L**IT***G*LTV*KF*IQ*KNVDL*   50

Xbub3      K-YQHAGPVLDCAFYDPT--HAWSGGLDHQ-LKMHDLNTDGDTVVGSHDAPI-RCVEYCP  112
hBub3      *-***T*A*********--*********-********QENL**T*****-******* 106
MMU67327                                *******QENL**T*****-*******  26
Bub3       QSLRYKH*L*C*N*I*N*DLQIYV*TVQGEI**VDLIGSPSFQALTNNE*NLGI*-RI*K  109

Xbub3      -EVNVIVTGSWDQTVKLWDPR---TPCNAG-TF-S-QP---DKVYTLSVSGDRLIVGTAG  162
hBub3      -****M**************---******-**-*-**---E***************** 156
MMU67327   -****M**************---******-**-*-**---E*****************A  76
Bub3       YGDDKLIAA***GLIEVI***NYGDGVI*VKNLN*NNTKVKN*IF*MDTNSS*****MNN  169

Xbub3      RRVLVW-DLRN-MGYVQQRRESSLKYQTRCIRAFPNKQ-GYVLSSIEGRVAVEYL-DPSL  218
hBub3      ******-****-************************-***************-***P 212
MMU67327   ******-****-************************-***************-***P 132
Bub3       SQ*-Q*FR*PLCEDDNGTIE**G****I*DVALL*KE*E**AC***D******FFD*QGD  228

Xbub3      EVQ-KKKYAFKCHRLKENNIEQIYPVNAVSFHNLHNTFATGGSDGFVNIWDPFNKKRLCQ  277
hBub3      ***-********************************************************  271
MMU67327   ***-**********************************************************LP  191
Bub3       DYNSS*RF**R****NLKDTNLA****SIE*SPR*KFLY*A****IISC*NLQTR*KIKN  288

Xbub3      FHRY-PTSIASLAFSNDGSTLAIAASYMYEMDDIDHPEDAIYIRQVTDAETKPK        330
hBub3      ****-*************T*****S********TE****G*F************SPCT    328
MMU67327   VPS*-*************T*****S********TE****G*F************ST      246
Bub3       *AKFNED*VVKI*C*DNILC**TSDD-TFKTNAAIDQTIELNASSIYIIFDYEN        341
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FIG. 2. Alignment of the amino acid sequences of *Xbub3*, *hBub3* (AF053304; Taylor and McKeon, 1998), MMU67327 (deposited in GenBank by Downs *et al.*, in press), and *Bub3* (M64707, Hoyt *et al.*, 1991). Conserved amino acids are represented by asterisks (*). The predicted protein of *Xbub3* showed 93.2% homology with hBub3, 91.0% homology with MMU67327, and 29.8% homology with Bub3.

used as a template for the PCR under the following conditions: 40 cycles at 94°C for 30 sec, 40°C for 2 min, 72°C for 30 sec. Arbitrary primer with a *Hin*d III site, 5′-AAGCTTCAGCGAA-3′, was used as upstream primer and oligo(dT) primer, 5′-AAGCTTTTTTTTTMA-3′, as downstream primer. Imaging of the polymerase chain reaction (PCR) products on acrylamide gel and isolation of specific cDNA bands were performed using the silver straining method specially developed for gene cloning (Shiraishi and Kinoshita, manuscript submitted).

Cloning and sequencing

Isolated and reamplified PCR products were inserted into the *Eco*RV site of the pBSKII(+) plasmid by the T-vector method (Marchuk *et al.*, 1990). Screening a full-length cDNA from an oocyte λgt10 cDNA library was performed using digoxygenin-labeled PCR fragments as a probe. Digoxygenin was detected by AP-conjugated anti-digoxygenin antibody and Attophos substrate (Boehringer Mannheim). Chemifluorescent signals were visualized by the Fluorimager (Molecular Dynamics). The nucleotide sequence of the cDNAs was determined using an ALFred DNA Sequencer (Pharmacia) with a fluorescent dye-labeled primer cycle sequencing kit (Amersham).

Quantitative RT-PCR

Total RNAs were extracted by the acid guanidine phenol–chloroform method from the embryos at various developmental stages. The first-strand cDNAs were synthesized with downstream primer and Reversetranscript I (Wako) from 1 μg of total RNA. Single-strand cDNA was subjected to the PCR under the following conditions: 30 cycles at 94°C for 30 sec, 55°C 30 sec, 72°C 1 min. The signals amplified by the PCR increased exponentially up to 40 cycles, so the amplified signals were not saturated under the conditions used. The sequences of the primers were 5′-ACTCATCTGTGCGCCTGTAT-3′ (upstream) and 5′-GTCCCATAGCTTCACGGTTT-3′ (downstream) for *Xbub3* and 5′-CGGGATAACATTCAGGGTATCACT-3′ (upstream) and 5′-ATCCATGGCGGTAACTGTCTTCCT-3′ (downstream) for XELHIS4 (*Xenopus* histone-H4). The PCR products were examined on a 3.0% agarose gel.

Whole-mount in situ *hybridization*

As a probe for *in situ* hybridization, antisense RNAs of *Xbub3* were generated by linearizing the vector with *Sma*I and transcribing with T7 RNA polymerase in the presence of

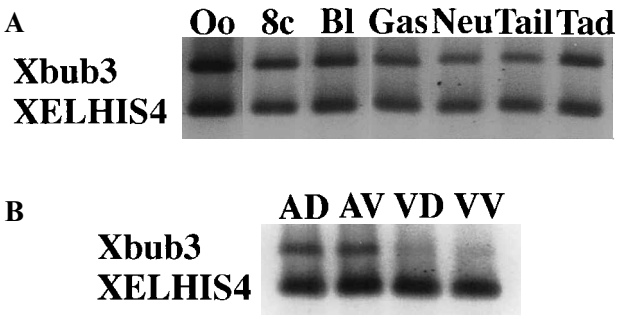

FIG. 3. Expression profile of *Xbub3*. Quantitative RT-PCR was performed using *Xbub3*-specific primers. Histone H4 (XELHIS4) was used for internal maker. **A.** Expression pattern of *Xbub3* during embryonic stages. Oo, oocyte; 8c, 8-cell-stage embryo; Bl, blastula (st. 7); Gas, gastrula (st. 12); Neu, neurula (st. 15); Tail, tailbud (st. 23); Tad; tadpole (st. 37). **B.** Localization of *Xbub3* transcripts in 8-cell-stage embryo. AD, animal–dorsal blastomeres; AV, animal–ventral blastomeres; VD, vegetal–dorsal blastomeres; VV, vegetal–ventral blastomeres.

digoxygenin-UTP (Boehringer Mannheim). Whole-mount *in situ* hybridization was performed according to the method of Harland (1991). As a minor modification of the method, follicle cells and vitelline membrane were removed with collagenase and trypsin before fixation of the oocytes. Some embryos were cleared in 2:1 benzyl benzoate/benzyl alcohol (BB:BA). After detection, some embryos were embedded in plastic resin and cut into serial sections with a thickness of 5 to 10 μm.

RESULTS

Cloning of Xbub3 *from maternal transcripts*

In order to identify a novel gene which is maternally transcribed and localized to a specific position within the oocyte, mRNAs were compared among four blastomere pairs of 8-cell-stage embryos using the differential display method. An animal blastomere-enriched cDNA fragment was subcloned from the differentially displayed PCR products. Using the digoxygenin-labeled PCR fragment as a probe, a 2940-bp cDNA clone was screened from the oocyte cDNA library. It was confirmed by Northern blot analysis that this clone corresponded to the full length of the transcript (data not shown). This clone contained an open reading frame (ORF) of 990 bp and a long 3′ untranslated region (Fig. 1). The ORF began 286 bp from the 5′ end of the sequence and encoded a predicted protein of 330 amino acids. According to the prediction of the beta sheet structures by the self-optimized prediction method (SOPM; IBCP-Web server analysis, Lyon, France) and WD-repeat consensus regular expression (Neer *et al.*, 1994), it is suggested that the predicted protein contains maximally six WD-repeat structures (Fig. 1).

A homology search by the BLAST program of the database of GenBank showed that this clone is a homolog of the human mitotic checkpoint gene *hBub3* (AF053304; Taylor and McKeon, 1998). Therefore, this clone was named *Xbub3*. A predicted protein of *Xbub3* showed high homology with human *hBub3* throughout whole region of the molecule (Fig. 2). Another homologous gene was obtained from mouse bone marrow-derived lymphohematopoietic stem cells (MMU67327; deposited in GenBank by Downs *et al.*, in press). This gene encodes a WD-repeat type I transmembrane protein, A72.5. Homology of *Xbub3* with *Saccharomyces cerevisiae Bub3* (M64707; Hoyt *et al.*, 1991) was extremely low (Fig. 2).

Developmental profile of Xbub3 *expression*

To examine the expression pattern of *Xbub3* during early development, total RNAs were isolated from eggs or embryos at various developmental stages, and quantitative RT-PCR was performed using *Xbub3*-specific primers. Active transcription of *Xbub3* was detected in oocytes (Fig. 3A). For examining the localization of the maternal transcripts in the early embryo, we quantitatively compared *Xbub3* mRNAs among four blastomere pairs in 8-cell embryos; animal–dorsal (AD), animal–ventral (AV), vegetal–dorsal (VD) and vegetal–ventral (VV) blastomeres. Quantitative RT-PCR showed that the maternal transcripts of *Xbub3* localized exclusively to animal blastomeres (Fig. 3B). This result is consistent with that of differential display. Transcripts of *Xbub3* were recognized throughout all developmental stages, and an increase of zygotic expression was observed from the tadpole stage (Fig. 3A).

Localization of Xbub3 *mRNAs*

Distribution of *Xbub3* mRNAs was examined by whole-mount *in situ* hybridization using a digoxygenin-labeled RNA probe. Localization of the transcripts of *Xbub3* was different from early oogenesis to the full-grown oocyte. During the early stages (Dumont I–III), active expression of *Xbub3* was recognized in the whole oocytes (Fig. 4A). The maternal transcripts gradually accumulated in the animal half during the late stages of oogenesis (Dumont IV–VI) (Fig. 4A) and finally localized in the perinuclear cytoplasm of the full-grown oocyte (data not shown). During cleavage stages, *Xbub3* transcripts also maintained their localization around the nucleus, exclusively in the animal blastomeres and the animal side of the vegetal blastomeres (Fig. 4B, C). In the dividing blastomeres, however, *Xbub3* mRNA showed a close association with chromosome movement (Fig. 4C–E). The perinuclear localization of *Xbub3* transcripts was not observed in the vegetal-half blastomeres.

After gastrulation, zygotic expression of *Xbub3* was observed in the whole ectodermal region. Especially enhanced expression was observed in the dorsoanterior region (Fig. 5A). The active expression of *Xbub3* was maintained in the neural region (Fig. 5B). Finally, expression of *Xbub3* was observed in the central nervous system, eyes, and branchial arches (Fig. 5C, D).

DISCUSSION

Xbub3, *a* Xenopus *homolog of* Bub3

In the present study, *Xbub3* was cloned as a gene that is maternally transcribed and localized to the animal half of the oocyte. *Xbub3* showed a low homology with the *Saccharomyces cerevisiae* mitotic checkpoint gene *Bub3* but had high homology with human *hBub3*. *Xbub3* has a characteristic WD-repeat

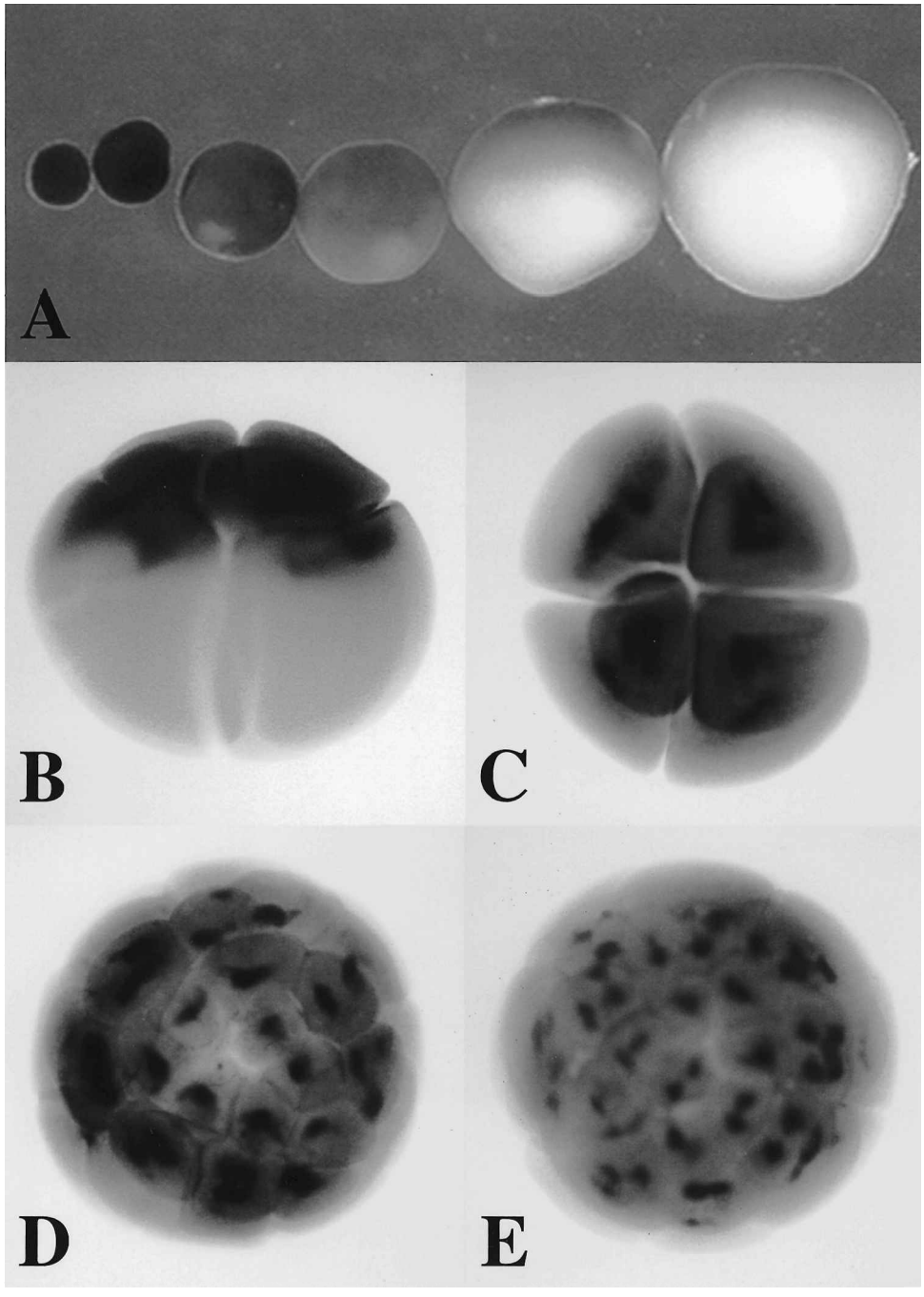

FIG. 4. Whole-mount *in situ* hybridization of *Xbub3*. **A.** Distribution of *Xbub3* mRNAs during oogenesis. Transcription of *Xbub3* occurs uniformly from stage I oocyte (left), and the transcripts gradually localize to the animal half by stage VI oocyte (right). **B–E**. Distribution of *Xbub3* mRNAs during cleavage stages. **B.** 8-cell-stage embryo (lateral view). **C.** 8-cell-stage embryo (animal view; interphase). *Xbub3* mRNAs are localized in the perinuclear cytoplasm. **D.** 32-cell-stage embryo (animal view; metaphase). *Xbub3* mRNAs are localized on metaphase chromosomes. **E.** 64-cell-stage embryo (animal view; anaphase). *Xbub3* mRNAs localize on anaphase chromosomes.

motif, the same as that of *Bub3* (Hoyt *et al.*, 1991) and *hBub3* (Taylor and McKeon, 1998). At first, *Bub3* has been identified as one of several yeast genes required for preanaphase delay in response to spindle disruption (Hoyt *et al.*, 1991). *Bub3* has a role as an extra copy suppressor of the *Saccharomyces cerevisiae Bub1-1* mutant (Hoyt *et al.*, 1991). *Bub1* encodes a protein kinase that can bind to and phosphorylate *Bub3* protein (Roberts *et al.*, 1994). Recent evidence suggests that the location of *Bub1* and *Bub3* in a signal transduction cascade is upstream of the other checkpoint genes, *Mad1* and *Mad2* (for a

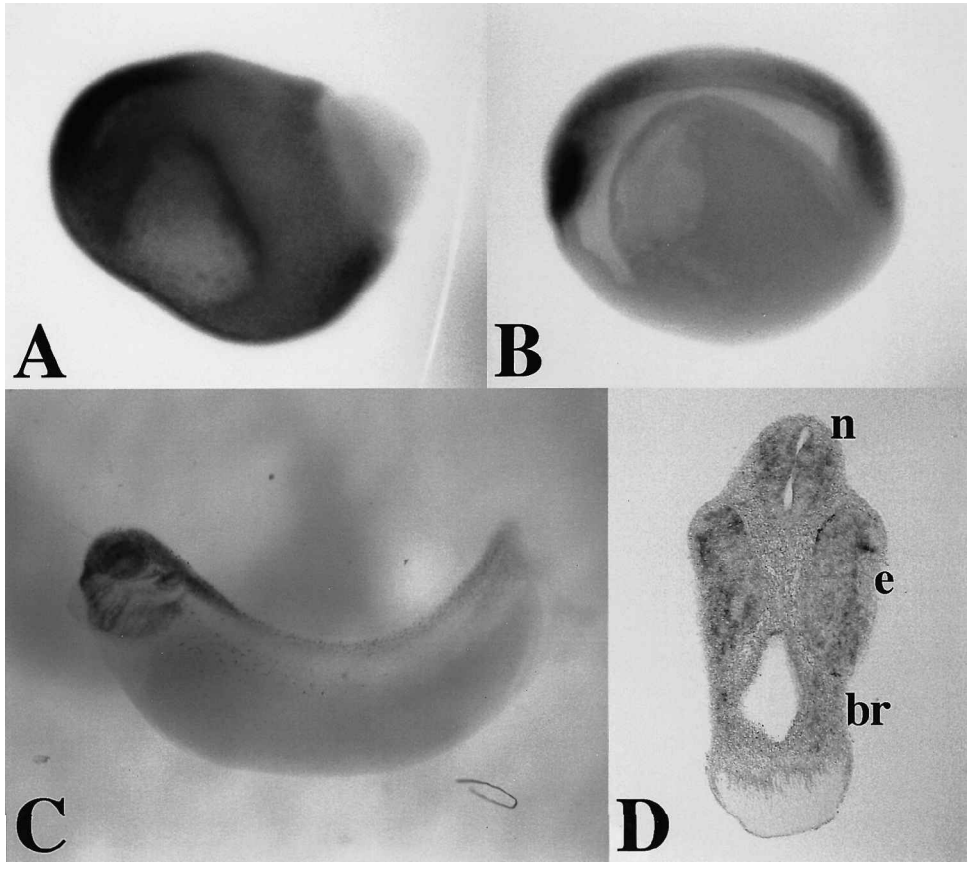

FIG. 5. Whole-mount *in situ* hybridization of *Xbub3*. **A.** Late gastrula (stage 12; lateral view). Zygotic expression of *Xbub3* occurs in the whole ectoderm, especially enhanced on the anterior neuroectoderm. **B.** Neurula (stage 15; lateral view). Restricted expression of *Xbub3* was observed in the neural region. **C.** Tadpole (stage 37; lateral view). **D.** Cross-section of the head region of the tadpole (stage 37; n, neural tube; e, eyes; br, branchial arches). Expression of *Xbub3* occurs exclusively in the central nervous system, eyes, and branchial arches.

review, see Wells, 1996). The *Xenopus* homolog of *Mad2* has been shown to localize to the kinetochore and to maintain MPF activity (Chen *et al.*, 1996). Therefore, *Xbub3* might play a role in the kinetochore-mediated mitotic checkpoint through the regulation of MPF activity.

Localization of Xbub3 *mRNAs*

Murine Bub1 protein localizes to the kinetochore using the N-terminal localization domain (Taylor and McKeon, 1997). Judging from the evidence that *Saccharomyces cerevisiae* Bub3 is phosphorylated by directly bound Bub1 kinase (Roberts *et al.*, 1994), it is likely that Bub3 also localizes to the kinetochore. Recently, a human homolog of the *Bub3* gene, *hBub3*, has been cloned. *hBub3* encodes a WD-repeat protein which localizes to kinetochores before chromosome alignment (Taylor and McKeon, 1998). Also, in the case of *Drosophila melanogaster*, the Bub3 homolog seems to localize to the kinetochore through Bub1 protein (Basu *et al.*, 1998). In the present study, maternally expressed *Xbub3* transcripts distributed in the perinuclear cytoplasm of mature oocytes and interphase blastomeres of cleavage-stage embryos. In metaphase blastomeres, however, *Xbub3* transcripts were recruited from blastomeres to their descendants by close association with the chromosome region. The close association between *Xbub3* mRNAs and chromosomes seems to ensure the localization of Bub3 proteins to the kinetochore.

WD-repeat protein

WD-repeat proteins have highly conserved repeating units, usually ending with Trp-Asp (WD). The characteristic WD-repeat motif was first reported in the β subunit of heterotrimeric G proteins (Fong *et al.*, 1986). Thereafter, the WD-repeat motif has been found in various proteins functioning in signal transduction, RNA processing, gene regulation, vesicular traffic, or regulation of the cell cycle (for a review, see Neer *et al.*, 1994). Most of these proteins seem to be regulatory, and there is a case that the WD-repeat region is fused with a protein kinase (Futey *et al.*, 1995). In some members of the WD-repeat superfamily, the WD-repeat region forms "β-propeller" folding (Garcia-Higuera *et al.*, 1996). Several WD-repeat proteins form multiprotein complexes, sometimes interacting with other proteins through the WD-repeat region (for a review, see Neer *et al.*, 1994). Bub3 seems to belong to the last group, as direct binding between Bub1 and Bub3 has been confirmed (Roberts *et*

al., 1994). A recent report suggests that human Bub3 localizes Bub1 to the kinetochore by binding to the kinetochore localization domains of Bub1 (Taylor and McKeon, 1998). In these studies, however, it is unknown whether the WD-repeat region of Bub3 was a binding site for Bub1. If the WD repeat of Bub3 is used for a protein other than Bub1, Bub3 may form multiprotein complexes. In *Xenopus* embryos, genes with special localization to the animal or vegetal half of the oocyte have been cloned by differential display method (Hudson *et al.*, 1996). Some of these genes have the WD-repeat motif. These genes belonging to WD-repeat superfamily may have some functional relation with each other.

Gene cloning by differential display method

Recent studies have reported novel genes with special localization within *Xenopus* eggs and embryos. An1 (Linnen *et al.*, 1993), An2 (Weeks and Melton, 1987a), and BMP-2 (Suzuki *et al.*, 1994; Hemmati-Brivanlou and Thomsen, 1995; Clement *et al.*, 1995) localize to the animal hemisphere in the oocyte. Vg1 (Rabegliati *et al.*, 1985; Weeks and Melton, 1987b), Xcat-2 (Mosquera *et al.*, 1993), Xwnt-11 (Ku and Melton, 1993), and Xlsirts (Kloc *et al.*, 1993; Kloc and Etkin, 1994) localize to the vegetal hemisphere. In most of these cases, the differential screening method has been used for cloning the genes. The present study used the differential display method to identify the *Xbub3* gene. A similar trial has been performed in the *Xenopus* oocyte, which also resulted in cloning of WD-repeat genes (Hudson *et al.*, 1996). In both cases, their maternal transcripts are abundant in the mature oocyte. Because the differential display method seems to amplify preferentially these abundant cDNA sources by PCR, removal of the abundant RNAs must be prerequisite for finding rare transcripts from RNA-enriched cells such as the oocyte.

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