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Exogastrula Formation in *Xenopus laevis* Embryos Depleted with Maternal XmN-Cadherin mRNA by Antisense S-oligo DNA

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***Xenopus* XmN-cadherin gene appears to have dual functions, since its mRNA is maternally provided in unfertilized eggs, once disappears almost completely during gastrula stage, then accumulates again specifically in neural tissues in later stage embryos. In the present experiment, we first followed the change in XmN-cadherin mRNA level during oogenesis by RT-PCR and showed that this mRNA exists from the earliest stage of oogenesis and at least one third of it is inherited as a maternal mRNA. We then carried out an experiment to deplete the maternal XmN-cadherin mRNA by injecting its antisense S-oligo DNA into full grown oocytes. When mRNA-depleted oocytes were matured *in vitro* and fertilized eggs obtained therefrom by host transfer technique were allowed to develop, embryos cleaved normally and developed until blastula stage. Such XmN-cadherin mRNA-depleted blastulae initiated invagination, but further involution did not take place, and exogastrulae were formed. These results suggest that the main function of maternally provided XmN-cadherin mRNA is to support cell movement or rearrangement required later during gastrulation, rather than to maintain adhesion of blastomeres during cleavage and blastula formation.**

Cadherins, Ca^{2+}-dependent cell adhesion molecules (1), have important roles in various formative movements during embryonic development. In *Xenopus* embryos, N- (2), E-(XTCAD1) (3, 4), EP(C)- (5, 6, 7) and XB(U)-cadherin (8, 9) have been reported to occur, each one of which is expected to have specific function(s) to promote early embryogenesis.

cDNA for XmN-cadherin has been cloned from *Xenopus* tailbud cDNA library in our laboratory (10). The total length of the cDNA was 3690bp and the open reading frame (ORF) coded for 922 amino acids. The amino acid of the intracellular domain of XmN-cadherin had high amino acid identity (79%) to *Xenopus* N-cadherin, and thus considered to be a novel member of N-cadherin gene family (10).

Northern blot analysis revealed that mRNA for XmN-cadherin was one of uniformly-distributed maternal mRNAs within the egg. The level of the mRNA in the embryo once decreased to the background level during gastrula stage, and zygotic transcription started only from neurula stage mainly in neural tissues such as retina, brain and spinal cord (10). Thus, mRNA for XmN-cadherin shows dual patterns of expression in *Xenopus* embryogenesis, suggesting that this cadherin has at least two different functions.As for the zygotically-expressed XmN-cadherin,it can be easily expected that its function is related to differentiation and/or maintenance of neural tissues, since the sites of the expression are restricted to neural tissues. However, as for the role played by maternal XmN-cadherin, no experimental approach has so far been carried out.

In the present experiment, we first examined the expression pattern of XmN-cadherin mRNA in oocytes at various stages. Then, we carried out experiment to obtain a clue to understand the role played by the maternal XmN-cadherin in early embryos. For this purpose, we depleted XmN-cadherin mRNA by injecting antisense S-oligo DNA into oocytes before maturation, and after obtaining fertilized eggs using host transfer technique (11), examined their early development. We report here that XmN-cadherin mRNA-depleted embryos cleave normally, reach blastula stage and initiate

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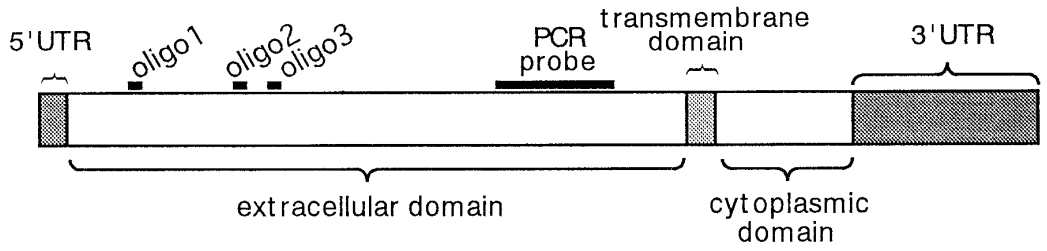

FIG. 1. Schematic structure of XmN-cadherin. Regions used for the present experiment for preparation of oligo DNAs (oligo1, oligo2, and oligo3) and for probe for PCR are indicated.

invagination, but fail to accomplish it and form exogastrulae.

MATERIALS AND METHODS

Oocytes. Ovary was excised from gravid females of *Xenopus laevis* anethetised by treatment with 0.2% MS222 (Nakarai. Co. Japan) for 15min. Ovary was rinsed with oocyte culture medium (OCM; 2.94g Leibowitz' L-15 medium and 0.16g bovine serum albumin dissolved in 400ml of distilled water) (11), and stage VI oocytes were collected and defolliculated manually using forceps. Oocytes were staged according to Dumont (12).

Oligonucleotides. The antisense and sense oligo DNAs used for microinjection were all 21-mers, which were designed based on the nucleotide sequence of cDNA for the extracellular domain of XmN-cadherin (11) (Fig.1) and prepared by Sawady Technology Co. Antisense oligo DNAs thus obtained were 5′-CAAATGCCCTGTGCTCCCGAA-3′ (oligo1: complementary to nucleotide no. 216-236 of XmN-cadherin ORF), 5′-CAGAGCGAATCAGCACAAGTT-3′ (oligo2: complementaly to nucleotide no. 584-604) and 5′-GGTCGTGTCACATTCATACGC-3′ (oligo3: complementaly to nucleotide no. 699-719). As oligo1 DNA, we prepared both antisense and sense S-oligo DNAs. Sense S-oligo DNA was 5′-TTCGGGAGCACAGGGCATTTG-3′ (identical to oligo1 part of cDNA). Antisense S-oligo DNA for EP-cadherin (oligo62:5′-CCACGTTCATTCTCAGAAACC-3′) was also prepared by Sawady Technology Co. according to Heasman *et al.* (13) and used as a control.

Microinjection, in vitro maturation and artificial fertilization. Oocytes were immersed in OCM, and injected into their cytoplasm with 1.6ng/oocyte of oligo DNA dissolved in 16nl of distilled water. Injected oocytes were cultured in OCM for1hr at 18°C for healing from the wound.

Maturation was induced in the injected oocytes by treating them with 2 μM progesterone (Sigma Co.) dissolved in OCM. Completion of maturation was confirmed by the appearance of a white spot after about 6 hrs at 18°C. Oocytes were then labelled with different colours. Thus,antisense oligo DNA-injected oocytes, sense oligo DNA-injected oocytes, and uninjected control oocytes were stained with Neutral red (red), Nile blue sulfate (blue) and their mixture (purple), respectively.

Oocytes with different colours were transferred into body cavity of a female which had been injected with a human gonadotropic hormone Gonatropin (Teikoku Zoki Co.) about 9 hrs before the transfer. Methods used for this host transfer technique were as described previously by Heasman *et al.* (11). About 2 hrs after the transfer, eggs were manually squeezed out of the frog. The host-transferred oocytes, now obtained as jellied fertilizable eggs, were inseminated with a sperm suspension. As will be described in Results and Discussion in details, efficiency of the fertilization of host-transferred eggs was usually much lower than that of naturally ovulated eggs. Fertilized eggs were dejellied in 2% L-cysteine-HCl (pH 8.0), and cultured in 0.1× Steinberg's solution at 21°C. Embryos were staged according to Nieuwkoop and Faber (14).

RNA extraction and RT-PCR. RNAs were extracted from oocytes and embryos by the Proteinase K-phenol method (10). RNAs were denatured, electrophoresed on 1% agarose formaldehyde gels, and filmed after stained with ethidium bromide.

To detect XmN-cadherin mRNA, we carried out RT-PCR, in which 5′-CCAGTCTACGGCAGCAGTGA-3′ (identical to the sequence from nucleotide number 1482 to 1501 of XmN-cadherin cDNA coding region) and 5′-ATCGATGGCGGTTATGTTTA-3′ (complementaly to the sequence from nucleotide number 1910 to 1929 of XmN-cadherin cDNA coding region) (Fig.1) were used as primers. For PCR, denaturation at 94°C for 1min, annealing at 60°C for 1min and elongation at 72°C for 2min were repeated for 35 times. By repeating the RT-PCR using different amounts of oocyte RNAs, it was confirmed that intensities of signals of reaction products obtained under the above conditions were proportional to the amount of imput RNAs, and therefore results with different RNA samples were mutually comparable.

RESULTS AND DISCUSSION

RNAs were extracted from oocytes at six different stages and from full grown oocytes which had been treated with progesterone for 3 and 6 hrs. As expected from the earlier study (15), the amount of RNA used for RT reaction increased as oocyte grew but did not change during maturation as seen by the amount of 18S and 28S rRNAs (Fig.2B). However,since the conditions for PCR were selected so that the signal obtained was roughly proportional to the input RNA and the amount of input RNA (one oocyte-equivalent RNA) was within the range of the quantitative determination (data not shown),and furthermore, the presence of varying amount of rRNA did not interfered with the detection of XmN-cadherin signal (data not shown), we determined the strength of the signals by densitometry as in Fig.2C. The mRNA signal occurred at a high level from stage I oocyte, and the level decreased gradually as oocytes grew and reached about one-third of the initial level at stage VI. The level of the mRNA in stage VI oocyte did not change greatly during maturation, as reported for most of maternal mRNAs (16, 17). These results show that XmN-cadherin mRNA occurs in oocytes from the previtellogenic stage at a relatively high level, and is inherited as maternal mRNA, although

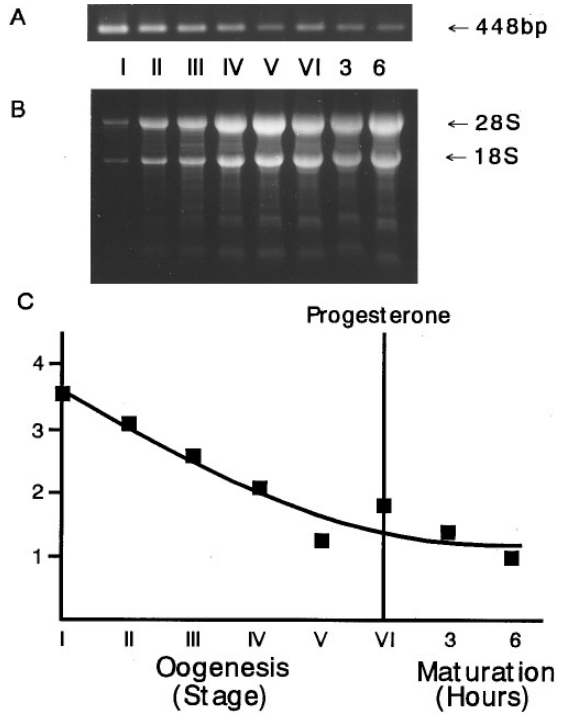

FIG. 2. Changes in the level of XmN-cadherin mRNA during oogenesis and oocyte maturation. RNAs were extracted from oocytes at 6 different stages (12) and the same batch of stage VI oocytes treated with progesterone for 3 and 6hr. A: Results of RT-PCR. I to VI indicates stage I to VI, and oocytes were treated with progesterone for 3hrs (3), 6hrs (6). B: Ethidium brommide staining of 18S and 28S (arrow heads) rRNAs on an agarose gel. The amount of mRNA loaded here was equivalent to one oocyte. C: Changes in the level of RT-PCR signal of XmN-cadherin mRNA throughout oogenesis and oocyte maturation as determined by densitometry of the signals in A. Three different experiments were carried out and representative data are shown.

more than a half of the mRNA disappears during oogenesis.

In order to obtain the clue to the function of the maternally-inherited XmN-cadherin, we searched the conditions to deplete of the mRNA by antisense oligo DNAs. We first microinjected different oligo DNAs designed for the extracellular domain of XmN-cadherin (antisense oligos 1, 2 and 3) into 2-cell stage embryos into both blastomeres (1 or 2ng/embryo). In embryos treated this way, however, only a slight decrease was observed in the level of XmN-cadherin mRNA at blastula stage as detected by RT-PCR (data not shown). It has been known that antisense oligo DNA to EP-cadherin mRNA injected into stage VI oocyte (11) degrades maternal EP-cadherin mRNA, in all the probability, due to digestion of double-stranded part by RNaseH-like activity which is abundantly present in oocytes (18, 19). We, therefore, adopted the methods to inject antisense oligo DNAs into oocytes and to obtain fertilized eggs from injected oocytes by using the host transfer technique (11) as stated above.

When we injected 1 or 2 ng of antisense oligo DNAs (antisense oligos 1, 2 and 3) into oocytes and after 8 hrs monitored the change in the level of the signal (448bp) of XmN-cadherin mRNA by RT-PCR, only oligo1 was found to effectively reduce the mRNA; the effect of oligo3 was weak and oligo2 was non-effective (Fig.3A).

Based on these results, we decided to use oligo1 hereafter, and prepared antisense, and in addition, sense S-oligo1 DNAs to test their effecs in oocytes, since S-oligo (phosphorothioated) DNA is known to be more stable, and therefore more potent, than usual oligo DNA in reducing maternal RNA (20). For comparison, we also tested here the effect of the injection of antisense S-oligo DNA to EP-cadherin mRNA, which was successfully used to eliminate maternal EP-cadherin mRNA by Heasman et al. (13). As shown in the results of RT-PCR carried out at 8hr after injection (Fig.3B), antisense S-oligo1 DNA was in fact far more effective than oligo1 DNA and both sense S-oligo1 DNA and antisense S-oligo DNAs for EP-cadherin mRNA were non-effective in reducing the level of the signal of XmN-cadherin. Based on these results we decided to use antisense S-oligo1 in the following experi-

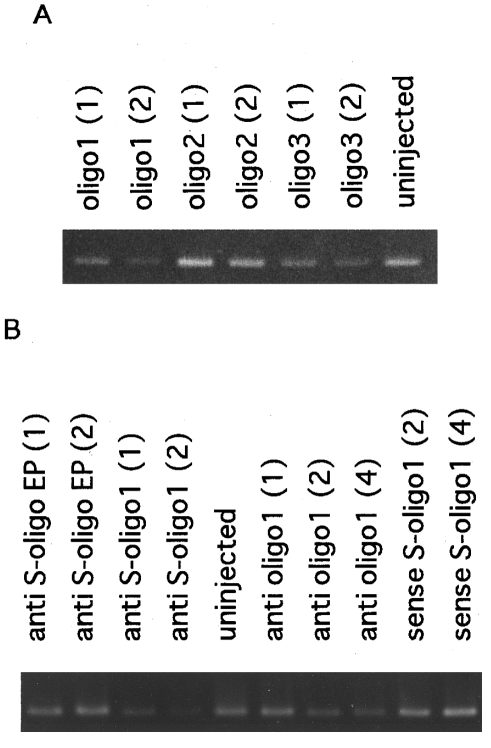

FIG. 3. Effect of microinjection of various oligo DNAs on XmN-cadherin mRNA level in full grown oocytes (stage VI). RNA from 1 oocyte was used for RT-PCR. The letter anti means antisense, and figures in parentheses indicate the amount of oligo DNA (ng) injected per oocyte. RNA equivalent to one oocyte was analyzed as in Fig.2.

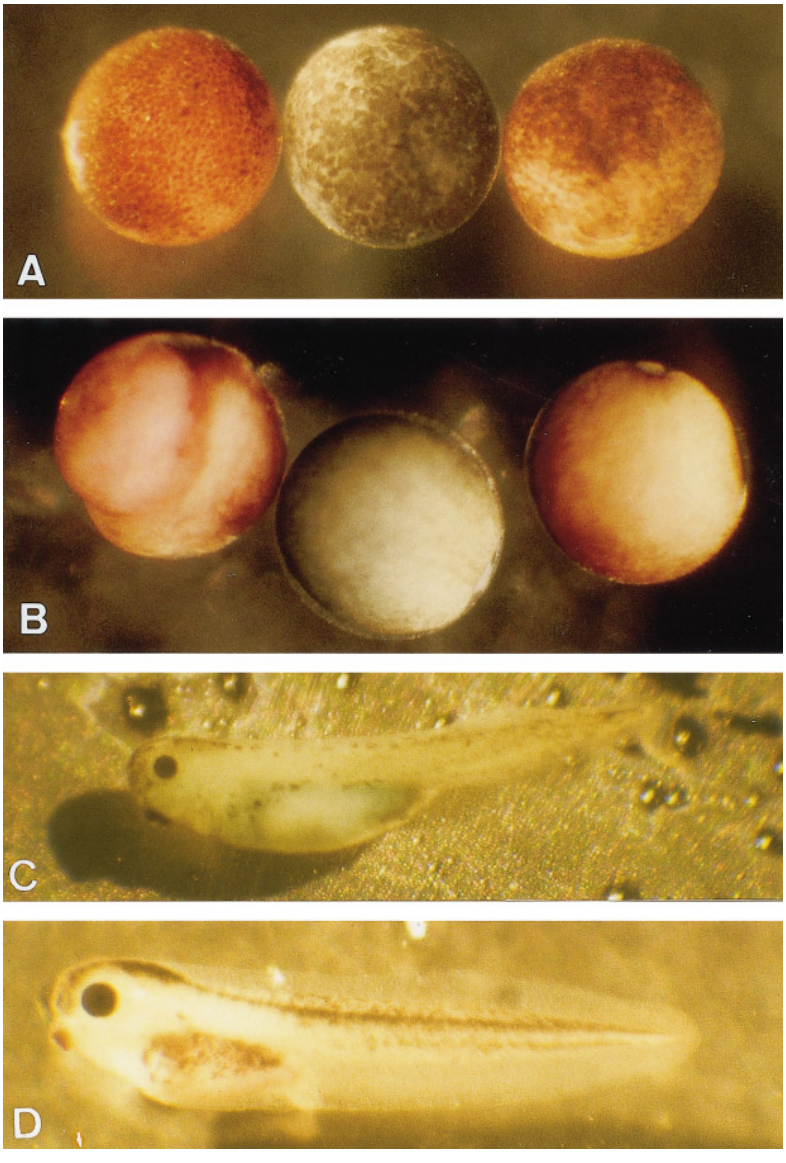

FIG. 4. Effect of microinjection of oligo DNAs on embryonic development. A: Blastula (stage8). From left to right, antisense S-oligoDNA-injected embryo (red), sense S-oligo DNA-injected embryo (blue), uninjected control embryo (purple). Each embryo showed normal appearance at this stage. In this case, the embryo in the middle (sense S-oligo DNA-injected) looks slightly retarded in development. However, this is simply because the timing of artificial fertilization was slightly retarded. B: Late gastrula (stage12). Antisense S-oligoDNA-injected embryo (left,red) could not gastrulate normally and formed exogastrula. Sense S-oligoDNA-injected embryo (middle, blue) and untreated control embryo (right) underwent normal gastrulation. C: Sense S-oligoDNA-injected embryo (stage 38 tadpole). D: Untreated control embryo (stage 42 tadpole). Scales are not identical in C and D.

ment as a specific and effective oligo DNA for depletion of maternal XmN-cadherin.

Oocytes were injected with either antisense S-oligo1 or sense S-oligo1 DNA at 1.6ng/oocyte, and after completion of *in vitro* maturation, stained with Neutral red (red) or Nileblue sulfate (blue), respectively. Uninjected oocytes as a control were also induced maturation *in vitro* and stained with a mixture of these dyes (purple). All the three groups of oocytes were transferred to the body cavity of a Gonadotropin-pretreated female, and after being ovulated, fertilized *in vitro*.

When we started the experiment, we noticed that fertilization rate of *in vitro* matured and host-transferred eggs was lower (average 50%) than that (average 70%) of naturally ovulated eggs. After the cortical rotation, which is a sign of fertilization, the percentage of eggs which underwent normal first cleavage was also considerably lower in the "host-transferred" eggs (average 50%) than in the normally ovulated eggs (average

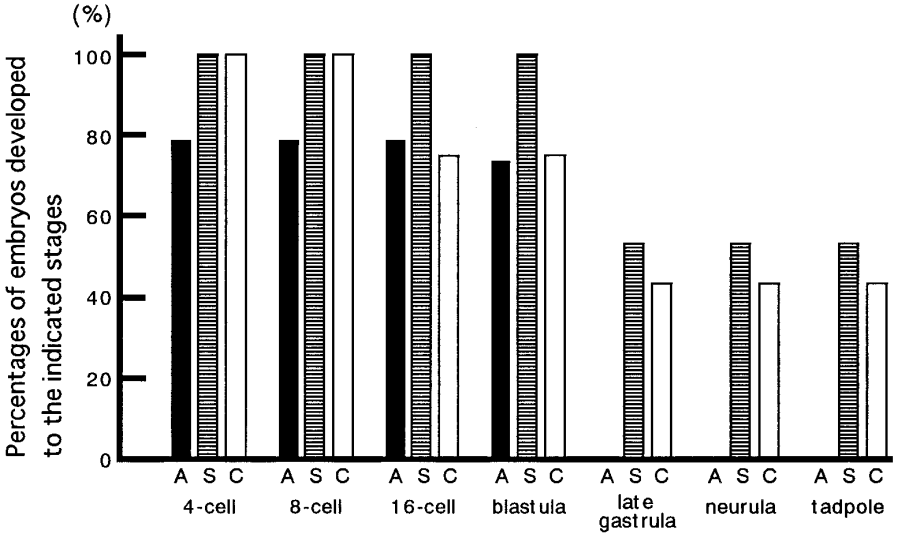

FIG. 5. A summary of the results of the experiments to test the effect of antisense S-oligo1 injection into oocytes. The results of the 5 experiments were essentially the same, and a typical set of the results is shown here. The number of total fertilized embryos used was 19, 15, and 16 for antisense S-oligoDNA-injected embryo(A), sense S-oligoDNA-injected embryo(S), uninjected control embryos(C), respectively. Embryos were examined at stage3 (4-cell), 4 (8-cell), 5 (16-cell), 8 (blastula), 12 (late gastrula), 23 (neurula) and 28 (tadpole), and percentage of the normal embryos in the total number of embryos which normally underwent first cleavage was determined at each stage.

90%). Nevertheless, there was no significant difference, on an average, in the percentage of the formation of normal blastulae among antisense S-oligo1-injected, S-oligo1-injected and uninjected embryos, all obtained by the host transfer technique. Probably, the lowered percentages of successful fertilization, first cleavage and subsequent development observed here for "host-transferred" embryos may be due to damages in eggs given during multi-step pretreatments, which included oocyte isolation, defolliculation, *in vitro* maturation, staining with vital dyes, host transfer and artificial fertilization. Nevertheless,it was also observed consistently that once fertilized eggs obtained by the host transfer technique underwent first or second cleavage, most of such embryos developed normallyat least until blastula stage.

Figure 4 shows the development of the three differently-treated embryos which underwent the first cleavage normally. In this experiment we confirmed that embryos which had been injected with antisense S-oligo DNA contained only a negligible amount of maternal XmN-cadherin mRNA, whereas embryos injected with sense S-oligo DNA and uninjected embryos contained a normal level of XmN-cadherin mRNA (data not shown). Fig.4A shows that three differently-treated embryos (antisense S-oligo1-injected, sense S-oligo1-injected, and uninjected embryos) cleaved normally and reached blastula stage. It appeared here that cell adhesion of blastomeres was not impared appreciably, since embryos appeared normal and were not dissociated into cells even when we removed the vitelline membrane and manually broken surface coat (data not shown). After blastula stage, however, while most of the sense S-oligo1-injected and uninjected control embryos developed further and reached yolkplug and then tadpole (Fig.4C,D) stages, all the antisense S-oligo1-injected embryos stopped development shortly after the initiation of gastrulation and formed exogastrulae (Fig.4B).

We carried out in total 5 separate experiments with essentially the same results. Figure 5 show a set of typical result. It is apparent here that although sense S-oligo1-injected embryos reached late gastrula stage, there was a significant decrease in the percentage of the embryos which developed further as normal embryos. However, since such reduction occurred also in uninjected control embryo, we assumed that the reduction observed here may be due again to the damage caused by the complex experimental procedures adopted. Thus, we concluded that although depletion of XmN-cadherin mRNA does not cause interference with cleavage, it induces inhibition of continued invagination, especially involution of mesodermal cells, thereby forming exogastrulae.

In the present experiment, we showed by RT-PCR that XmN-cadherin mRNA occurs in oocytes from the earliest stage of oogenesis and although about two-third of it disappears during oogenesis, it is inherited as maternal mRNA in the egg. Furthermore, using antisense S-oligo DNA we demonstrated that depletion of the maternal XmN-cadherin mRNA inhibits the formation of normal gastrula and induces exogastrula for-

mation, although it does not interfere with cleavage, blastula formation and initiation of gastrulation. Based on these results, we suggest that XmN-cadherin expersses its function not during cleavage and blastula formation but during invagination. Recently, it has been suggested that *Xenopus* maternal cadherins may have roles not only for the maintenance of cell adhesion but also for cellular movements (8, 21). Therefore, it appears that maternal XmN-cadherin may function in early embryos as one of such a type of cadherins.

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