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Two Types of Na^+/K^+-ATPase Alpha Subunit Gene Transcript in Embryos of the Sea Urchin, *Hemicentrotus pulcherrimus*

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ABSTRACT—We have characterized the nucleotide sequence of Na^+/K^+-ATPase alpha subunit (NKA) cDNA in embryos of the sea urchin, *Hemicentrotus pulcherrimus*. The primer extension experiments showed that the sea urchin NKA gene generated multiple lengths of transcript. To obtain the 5'-ends of the transcripts, we isolated cDNA clones by the rapid amplification of cDNA ends (RACE). These clones were classified into 2 types on the basis of their 5' leader sequences. The sequences of the clones were identical except their 5' leaders. By Northern blot analysis, 1 of the 2 types of transcripts was always detectable in sea urchin embryos during early development, and another was not detected before the morula stage. Genomic PCR demonstrated that the two 5' leaders were coded by different exons separated by an intron in a single gene. These results show that the transcripts coding 2 isoforms were expressed from a single gene.

INTRODUCTION

Na^+/K^+-ATPase is a known membrane-binding protein responsible for the active transport of Na^+ and K^+ (Sweadner, 1989; Vasilets and Schwartz, 1993). This enzyme consists of an alpha and beta subunit. In many species, it was reported that the tissue-specific isoforms of both subunits exist and that each isoform is encoded by a single gene (Shull and Lingrel, 1987; Lingrel *et al.*, 1990).

We have isolated cDNA clones coding 1 form of the Na^+/K^+-ATPase alpha subunit (NKA) by screening cDNA libraries of the sea urchin, *Hemicentrotus pulcherrimus* (Mitsunaga-Nakatsubo *et al.*, 1996). This gene is a single copy gene (Yamazaki *et al.*, 1997) as in the case of other species. Multiple isoforms of NKA, other than these, have not been identified by screening cDNA libraries of sea urchin embryos at the gastrula and prism stages, although it is not clear whether other isoforms, not expressed in these stages, are encoded in the sea urchin genome. In NKA, isoform- and species-specific regions are found in the N-terminal domain and the region just before the fluorescein 5'-isothiocyanate (FITC)-binding site (Lingrel *et al.*, 1990). The sea urchin NKA cDNA reported previously (Mitsunaga-Nakatsubo *et al.*, 1996) was identified as alpha III on the basis of the sequence before the FITC-binding site, although the N-terminal domain is not similar to any isoform known in other species. Here we isolated new clones by the RACE method, including the different 5' leaders and identical downstream sequences of the previous cDNAs.

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MATERIALS AND METHODS

DNA and RNA

Genomic DNA was prepared from sperm of the sea urchin, *Hemicentrotus pulcherrimus* by phenol/chloroform-extraction. Total RNA was extracted with ISOGEN (Nippon Gene) from unfertilized eggs, 16-cell embryos, morulae, early blastulae (prehatched), hatched blastulae, mesenchyme blastulae, and gastrulae.

Primer extension

Primer extension was performed using the Primer Extension Systems (Promega). A primer for reverse transcription (5'-ATTGCGTCCATCATCAGGGA-3') was synthesized to bind to approximately 100 bp downstream of 5'-end of the cDNA, as reported previously (Mitsunaga-Nakatsubo *et al.*, 1996). The ^{32}P-labeled primer was hybridized to 10 μg of total RNA extracted from mesenchyme blastulae and the hybrid was reverse transcribed. The reverse transcript and the ^{32}P-labeled ϕx174-*Hin*fI digests were separated by electrophoresis on a 7% acrylamide/7M urea gel. The gel was dried and autoradiogramed by exposure to the X-OMAT film (Kodak) overnight at -80°C.

DNA blot analysis

The reverse transcript by the primer extension with a nonlabeled probe was transferred to a nylon membrane, Hybond-N (Amersham). The two-type-detectable probe (the cDNA fragment between the type I 5'-end and *Bgl* II site)and the type II 5' leader-specific probe (the cDNA fragment between the type II 5'-end and *Sac* I site) were hybridized to the membrane in 5 × SSPE/5 × Denhardt's/0.5% SDS overnight at 65°C and then washed in 0.1 × SSC/0.1% SDS at 65°C. The filter was exposed to the X-OMAT film (Kodak) for 3 days at -80°C.

RACE

For cloning of the cDNA 5'-end, we performed the RACE (Frohman *et al.*, 1988) by oligo(dC)-tailing substituted for oligo(dA)-tailing (Mitsunaga-Nakatsubo *et al.*, 1996). The cDNAs cloned were

sequenced with the Sequenase™ version 2.0 7-deaza-dGTP kit (United States Biochemical Corp.).

Northern hybridization

Total RNA (5 μg), extracted from embryos at the several stages mentioned above, was electrophoresed on a formaldehyde/agarose gel as described by Sambrook *et al.* (1989). RNA in the gel was transferred to a nylon membrane, Hybond-N (Amersham), by the capillary-transfer method. Type I-specific oligonucleotide (5'-CCGCTTTTCTCTACACGGGCAGCCATCG-3') and type II-specific oligonucleotide (ALT4; 5'-TGAGCTTGAATAGTTATCTTGAGCAAGA-ACCC-3') were ^{32}P-labeled with T4 polymerase (Takara Shuzo). Each probe was hybridized with the nylon membrane in 5 × SSPE/5 × Denhardt's reagent/0.5% SDS at 55°C. The membrane was finally washed in 2 × SSC/0.1% SDS at 55°C. The signals of probes were detected by an imaging analyzer, BAS-2000II (Fuji Photo Film Co., Ltd.).

Genomic PCR

Approximately 500 ng of DNA prepared as described above was used as a template for PCR with the Expand™ Long Template PCR System (Boehringer Mannheim). Type I 5'-end-specific sense primer ALT2 (5'-TTCATTGATGATCACGATGGCTGCCCGTGT-3'), type II 5'-end-specific sense primer ALT1 (5'-GGGAGGTGCTTCAGTCACGG-AGGTTGCT-3'), type II 5'-end-specific antisense primer ALT4, and common antisense primer ALT3 (5'-CTGTACTGCAACACGGTAGCT-TTCCCAC-3') were synthesized and used for PCR in combinations of ALT1/ALT3, ALT2/ALT3 or ALT2/ALT4. Thermal cycle : series of 20 sec at 94°C and 10 min (ALT1/ALT3 and ALT2/ALT3) or 15 min (ALT2/ALT3) at 68°C was repeated 30 times with extension for 15 sec at 68°C every cycle. The PCR products were detected by electrophoretic analysis on a 0.5% agarose gel.

RESULTS

Figure 1A shows the autoradiogram of primer extension. As is shown in lane 1, multiple bands were generated from 10 μg of total RNA extracted from sea urchin embryos at the mesenchyme blastula stage. Three or more dense bands, represented by an asterisk, were products of the expected size, as determined from the cDNA characterized previously (Mitsunaga-Nakatsubo *et al.*, 1996). However, the 2 bands marked by arrows were larger in size. Then, cDNA clones obtained by the RACE method were used to determine the structures of cDNA 5'-end regions. Several cDNA clones thus obtained were sequenced and they were classified into 2 types (type I and II) on the basis of their 5' leader sequences. There was no cDNA containing both sequences for type I and II. The type I 5' leader was almost the same as that of the cDNA reported previously (Mitsunaga-Nakatsubo *et al.*, 1996). Partial sequences of the 2 types of cDNAs are shown in Fig. 2. The type II 5' leader was longer than the type I 5' leader, although the deduced amino acid sequence of type II was shorter than that of type I. Except their 5' leader sequences which were identical (downstream from the positions marked with 2 arrows in Fig. 2).

In each of the two 5' leaders obtained by RACE, the length differed slightly among clones. It seems that transcription initiation sites for each type are somewhat variable but transcripts involving each 5'-end are generated. Because of the variability in the transcription initiation sites of each 5' leader-containing mRNA, multiple bands were assumed to be generated by primer extension (Fig. 1A). The reverse transcripts using the same primer for primer extension without labeling were transferred to a nylon membrane and hybridized with a probe that detects both types, and another probe specific for type II 5' leader. The result of the hybridization is shown by the autoradiogram in Fig. 1B. The former probe detected all of the RT products (lane 1) and the latter probe bound to 2 bands (lane 2) which corresponded to the sizes of those shown by arrows in Fig. 1A. These indicate that the 2 primer extension products, represented by arrows in Fig. 1A, are the type II 5' leaders. All of the bands with an asterisk (Fig. 1A) have products containing the type I 5'-end varieties.

Northern blot analysis was performed using 2 probes, the type I specific oligomer (ALT5) and the type II specific oligomer (ALT4). Their binding sites are shown in Fig. 2. From 10 μg of total RNA extracted from unfertilized eggs (U), 16-cell embryos (16), morulae (Mo), early blastulae (eB), hatched blastulae (hB), mesenchyme blastulae (mB), and gastrulae (G), the type I transcripts were detected with ALT5 (Fig. 3A). The expression of the type I mRNA was insignificant in embryos before the early blastula stage and suddenly became evident at the

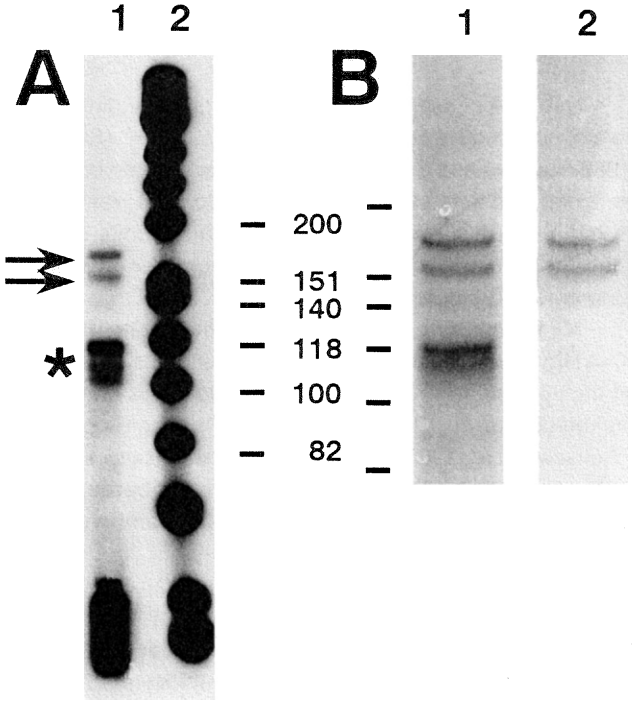


Fig. 1. (**A**) Analysis of NKA gene transctiption start site by the primer extension method. Reverse transcripts were loaded in lane 1 and molecular size marker (ϕx174-*Hin*f I digests) in lane 2. An asterisk shows the reverse transcripts expected for the 5' leader of the cDNA previously reported (Mitsunaga-Nakatsubo *et al.*, 1996). Two arrows reveal larger fragments of the 5' leaders found in this study. Molecular sizes are shown on the right side. (**B**) DNA blot analysis. The reverse transcripts on nylone membranes were hybridized to the probe which detects both types of transcript (lane 1) and the type II-specific probe (lane 2). Horisontal bars represent molecular sizes of 200 bp, 151 bp, 140 bp, 118 bp, 100 bp and 82 bp.

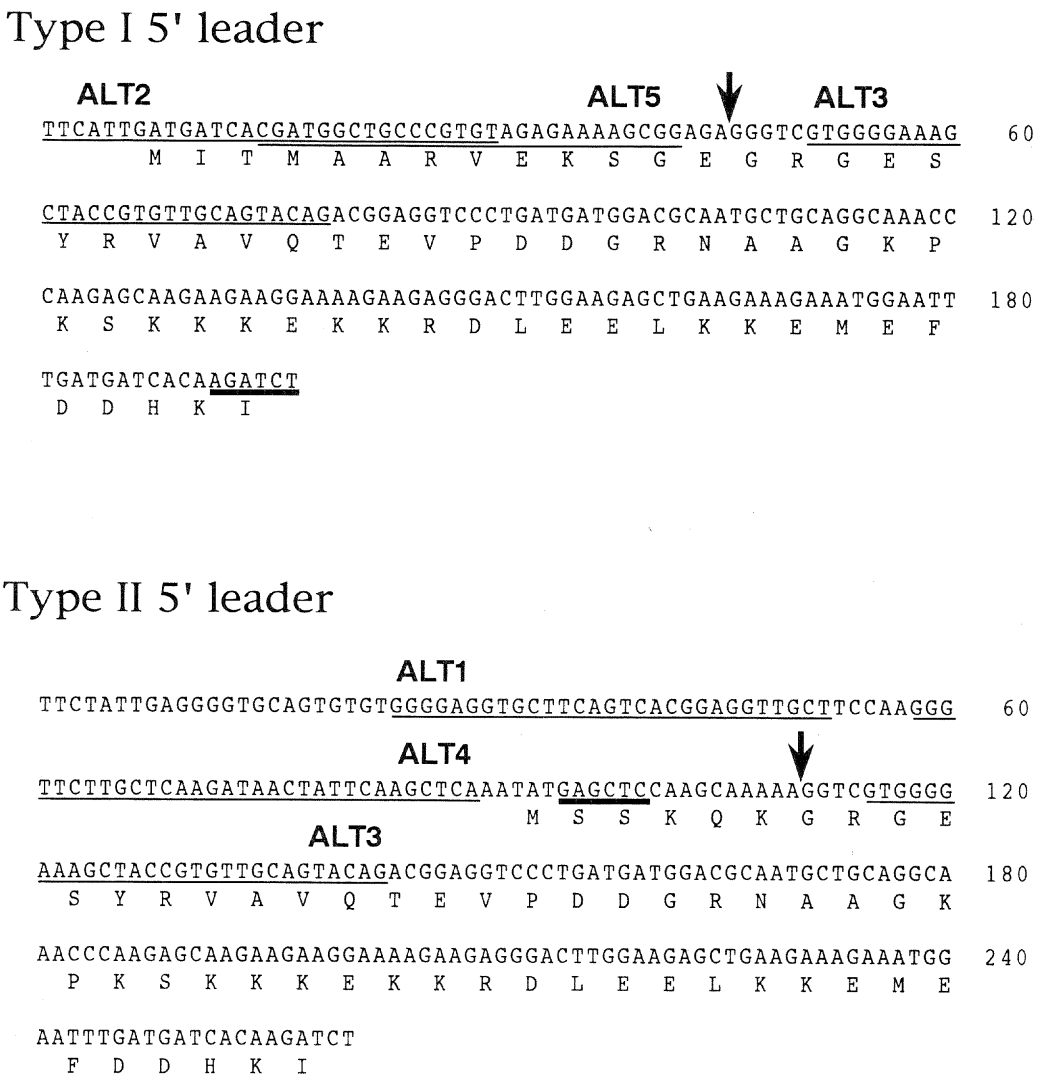
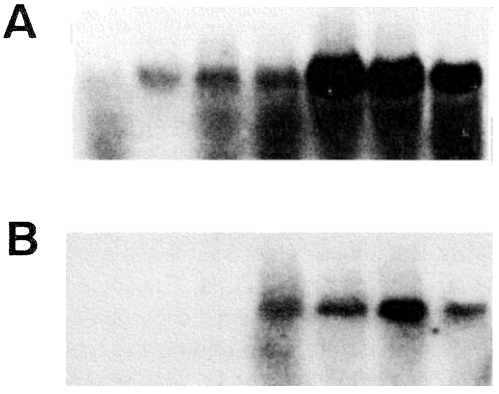


Fig. 2. Sequences of the type I 5' leader and the type II 5' leader. Nucleotide sequences are shown by small letters and the deduced amino acid sequences are shown in capital letters. Arrows show the junctions downstream which are identical in both types of transcript. The numbers of nucleotide in the cDNAs are shown at the right end of every line. Positions of primers for PCR (ALT1, ALT2, ALT3 and ALT4) and a probe for Northern hybridization (ALT5) are underlined. Bold underlined parts are recognition sites of *Sac* I (GAGCTC) and *Bgl* II (AGATCT).



hatched blastula stage. The type II mRNA, hybridized to ALT5, was undetectable in unfertilized eggs, 16-cell embryos, and morulae (lanes U, 16 and Mo in Fig. 3B), but was detected in embryos after the early blastula stage (lanes eB, hB, mB and G). The type II mRNA was faintly detected by RT-PCR from embryos before the morula stage (data not shown). After the blastula stage, the type I mRNA was abundant in comparison with the type II mRNA.

Genomic PCR was carried out by use of 4 primers, ALT1 (the type II 5' leader-specific forward primer), ALT2 (the type I

Fig. 3. Northern blot analysis detecting type I and II sea urchin NKA transcripts. In each lane, 5 μg of total RNA extracted from unfertilized eggs (U), 16-cell embryo (16), morula (Mo), early blastula (eB), hatched blastula (hB), mesenchyme blastula (mB) and gastrula (G) were loaded. The type I 5'-leader was specifically detected by ALT4 (A), and type II was detected by ALT5 (B).

5' leader-specific forward primer), ALT3 (the common downstream-specific reverse primer) and ALT4 (the type II 5' leader-specific reverse primer). Figure 4 shows the PCR products separated on an agarose gel. By combination of ALT2 and ALT3, fragments of over 10 kb were produced (lane 1) although 2 major fragments of smaller sizes (approximately 4 kb and 5 kb) were amplified by ALT1 and ALT3 (lane 2). Aliquots of the PCR product with ALT2 and ALT3 were subjected to secondary PCR with a combination of ALT1 and ALT3 (lane 3). The 2 fragments for the secondary PCR product were identical in size to those of the primary PCR product with ALT1 and ALT3 (lane 2). These indicate that an ALT1-binding site was located between the binding sites of ALT2 and ALT3 in the genome. However, from aliquots of the PCR product with ALT1 and ALT3 (lane 2), no secondary PCR product was generated with a combination of ALT2 and ALT3 (data not shown). Two fragments of the same sizes (approximately 8 kb and 9 kb) were amplified with ALT2 and ALT4 from the genome (lane 4) and ALT2/ALT3-PCR (lane 5). As expected from the cDNA sequences (Fig. 2A and B), short segments of exons were included in the PCR products of ALT1/ALT3 and ALT2/ALT4. The ALT1/ALT3 product probably involves a fragment between the ALT1-binding site and the exon-junction (an arrow in Fig. 2B) at one end, and between the junction and the ALT3-binding site at the other end. The product with ALT2 and ALT4 seems to include a segment between ALT2-binding site and the junction (an arrow in Fig. 2A) at one end, and between the type II 5'-end and the ALT4-binding site at the other end. These products probably involve introns of 4-5 kb and 8-9 kb, respectively.

This suggests that the two 5' leaders are coded on separated exons and that the exon coding the type I 5' leader was located upstream of the type II 5' leader-coding exon. The common exon for the 2 types is located downstream of the type II 5' leader-coding exon following another intron. The lengths of the 2 introns, interspersed between the exons coding the type I and II 5' leader and the common sequence, were variable among alleles probably due to an intron-length polymorphism as reported by Wydner *et al.* (1994). In the sea urchin NKA gene, some introns generate polymorphism in length with variable numbers of tandem repeats (Yamazaki *et al.*, 1997). For the genomic PCR template, the sea urchin genome extracted from sperm was used, and it involved both alleles. Two bands detected in the PCR products (Fig. 4) seems to be fragments amplified from 2 alleles.

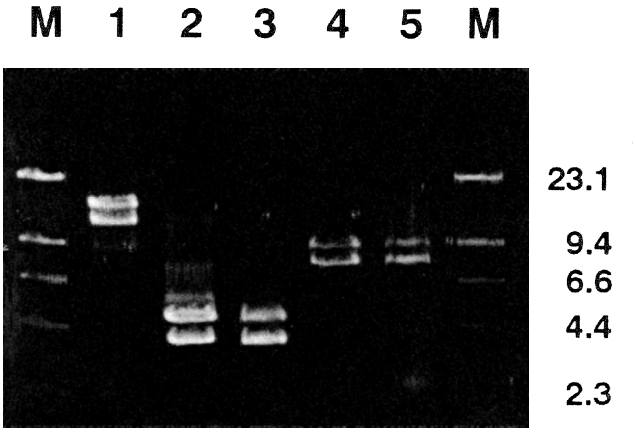


Fig. 4. Genomic PCR products detected by electrophoresis. DNA extracted from sperm were subjected to PCR with ALT2 and ALT3 (lane 1), ALT1 and ALT3 (lane 2) or ALT2 and ALT4 (lane 4). Aliquots of the PCR products in lane 1 was re-amplified with ALT1 and ALT3 (lane 3) or ALT2 and ALT4 (lane 5). M: DNA size marker, λ-*Hin*d III (Molecular sizes of these fragments are shown on the right side).

DISCUSSION

In embryos of the sea urchin, *Hemicentrotus pulcherrimus*, cDNAs of the NKA gene transcripts obtained by the RACE method were found to involve several different 5'-ends. On the basis of their 5' leader sequences, NKA gene transcripts were classified into 2 types, type I and type II. The type II 5' leader was larger than that of the type I. The deduced amino acid sequences of their N-terminal domains differed in size and sequence from each other, though the sequences downstream from these domains were identical in the 2 types. These observations suggest that 2 isoforms of NKA exist in sea urchin embryos. It has been reported that a common sequence downstream of the 5' leader in the 2 types is encoded by a single gene (Yamazaki *et al.*, 1997).

Genomic PCR, carried out by the use of ALT2, the type I 5' leader specific forward primer and ALT3, the common downstream specific reverse primer, generated fragments which were larger in size than the PCR products with ALT1, the type II 5' leader specific forward primer and ALT3. Secondary PCR with a combination of ALT1 and ALT3, performed on the products with ALT2 and ALT3, produced fragments similar in size to the products from the genome with ALT1 and ALT3. Secondary PCR with ALT2 and ALT3 on the genomic PCR products generated using ALT1 and ALT3, did not produce any detectable fragment. The products of genomic PCR with ALT2 and ALT4, the type II 5' leader specific reverse primer, were almost the same in size to those generated by secondary PCR with ALT2 and ALT4 of the genomic PCR products formed with ALT2 and ALT3. These results indicate that a binding site for ALT1, specific to the type II 5' leader sequence, exists between the binding sites of ALT2, specific to the type I 5' leader, and ALT3, specific to the common downstream sequence in the genome.

Genomic PCR products with ALT1 and ALT3 contained short segments coding the type II 5' leader and the common downstream sequence, and those with ALT2 and ALT4 involve short segments of the type I and II 5' leaders. These short segments are probably a part of exons coding the type I and II 5' leaders as well as the common downstream sequence, respectively. Sequences other than these exons in the PCR products are thought to be introns. Thus, it is likely that the NKA gene, reported to be a single copy gene (Yamazaki *et al.*, 1997), contains the exon coding the type II 5' leader between exons coding the type I 5' leader and the common

downstream sequences, which are separated by introns.

On the basis of NKA gene structure, it seems likely that expression of type II NKA is due to elimination of the type I 5'-leader-coding sequence by splicing from the transcript including both types of 5' leaders, or initiation of transcription at the type II 5' leader-coding exon, existing downstream of the type I 5' leader-coding exon in the single copy NKA gene. Some consensus sequences of *cis*-elements were found in an intron between the type I and II 5' leader-coding exons. Though the function of this region has not been identified, it is now under investigation, it is expected that a promoter exists upstream of the type II 5' leader-coding exon to initiate the expression of type II NKA. On the other hand, it is simply assumed that a transcript might contain a sequence corresponding to the type II 5' leader between the type I 5' leader and the common downstream sequence, if transcription is initiated at the exon coding the type I 5' leader. However, cDNAs obtained by the RACE method involved only one sequence of the type I or II 5' leader. Thus it is probable that the type II 5' leader-coding region is eliminated by splicing from a transcript including both types of 5' leaders, being accompanied by elimination of the structures derived from introns upstream and downstream of the type II 5' leader-coding exon.

These assumptions are made based on sequences of PCR and RACE products and should be confirmed via the NKA gene and mRNA coding type I and II NKA. However, these observations strongly suggest that 2 NKA isoforms are expressed due to "alternative promoters", a pattern of alternative splicing (Breitbart *et al.*, 1987). Several isoforms of NKA are found in other species and each of the NKA isoforms is encoded by a single gene (Lingrel *et al.*, 1990). There are no reports showing that transcripts of the NKA gene are processed by alternative splicing to produce mRNAs for NKA isoforms, although isoforms of other ATPases, such as Ca^{2+}-ATPase (Keeton *et al.*, 1993) and H^+-ATPase (Hernando *et al.*, 1995), are generated by alternative mRNA splicing.

Previously, it was reported that expression of the sea urchin NKA gene is augmented in a period of development between the mesenchyme blastula and the early gastrula stage (Mitsunaga-Nakatsubo *et al.*, 1993). Northern blot analysis in the present study indicated that type I NKA mRNA expression was maximum at the hatched blastula stage and expression of the type II mRNA became detectable at the early blastula stage. Between these works, the developmental stage of maximum expression of this gene is different, to some extent, although the probe for the Northern blot in the previous work, which detects both types of the transcripts, is different from the probes in this study. These discrepancies are now under investigation. Though the developmental stages for maximum expression of the NKA gene differed among these s studies, these studies show an augmentation of NKA gene expression during early development. The results also suggest that alternative splicing generates 2 types of NKA mRNA and this is activated in embryos during early development. Alternative splicing of NKA isoform expression is expected to be a tool for analysis of cell differentiation in early development.

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